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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/867, 15/11, 5/10

A1

(11) International Publication Number: WO 00/00629

(43) International Publication Date: 6 January 2000 (06.01.00)

(21) International Application Number: PCT/KR99/00334

(22) International Filing Date: 24 June 1999 (24.06.99)

(30) Priority Data:

1998/24478	26 June 1998 (26.06.98)	KR
1999/23398	22 June 1999 (22.06.99)	KR

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

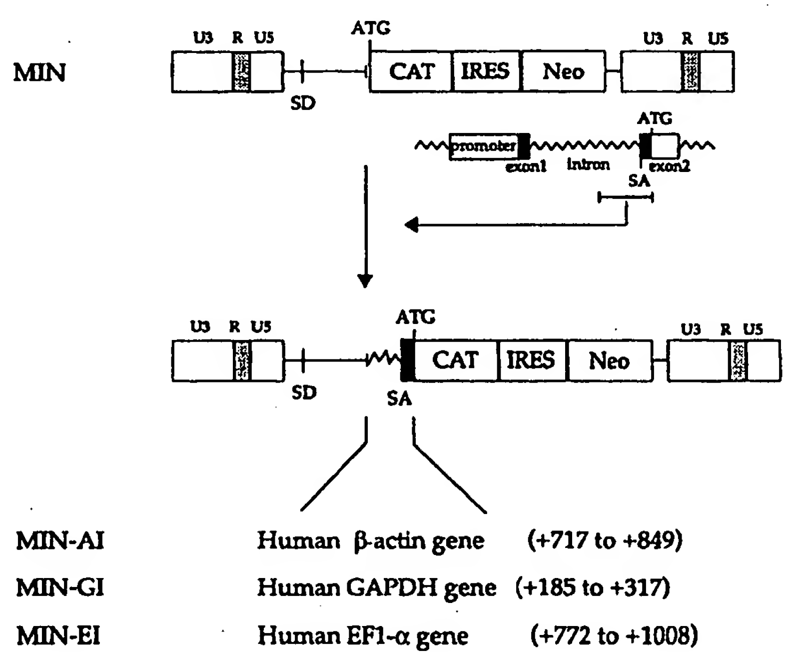
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description.

(54) Title: HIGH EFFICIENCY RETROVIRAL VECTORS THAT CONTAIN NONE OF VIRAL CODING SEQUENCES

(57) Abstract

The present invention relates to improved retroviral vectors for gene therapy. In this invention, retroviral vectors with higher safety and efficiency are constructed from MLV-based starting vectors, MON and MIN. The improved vectors have following features: 1) sequences corresponding to MLV-derived *pol* gene are completely deleted in the vectors, avoiding homologous recombination which has been a baffling problem in conventional retroviral vectors, 2) a heterologous intron, splicing acceptor and/or non-coding sequence are/is inserted into the upstream position of cloning site, maximizing the expression of a foreign gene through efficient splicing, 3) the vectors contain either the full-length U3 sequence of 5' LTR or a strong heterologous promoter instead, permitting the abundant production of RNA, 4) either IRES (Internal Ribosomal Entry Site) or internal SV40 minimal promoter is inserted into the downstream position of cloning site, enabling the simultaneous expression of two or more foreign genes. Since the improved retroviral vectors of this invention turn out to be safe and to express the foreign gene efficiently, they are useful for gene therapy and the like.



Serial No. 09/904,782

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HIGH EFFICIENCY RETROVIRAL VECTORS THAT CONTAIN NONE OF VIRAL CODING SEQUENCES

FIELD OF THE INVENTION

The present invention relates to improved retroviral vectors for gene therapy. Particularly, this invention relates to safe and efficient retroviral expression vectors, where all of the retroviral genes, i.e. *gag*, *env* and *pol*, are deleted completely; where a heterologous intron, splicing acceptor, and/or non-coding sequence are/is inserted into the upstream position of cloning site for a foreign gene; where a heterologous internal promoter or an internal ribosomal entry site (hereinafter, referred to as "IRES") is inserted into the the downstream position of the cloning site; and where the full-length U3 sequence of 5' LTR (Long Terminal Repeat) or a strong heterologous promoter controls the expression of the foreign gene.

BACKGROUND

Retroviral vectors has been used for gene therapy more frequently than any other vector, being employed in more than 50% of the approved clinical protocols worldwide (Wiley - Journal of Gene Medicine Website; http://www.wiley.co.uk/genetherapy). Although Murine

Leukemia Virus (hereinafter, referred to as "MLV")-based vectors are used dominantly, there are still many problems with the retroviral vectors in clinical use. The most serious problem is their safeties; retroviral vector is one of the viral vector and thus may be converted in cells into replication-competent retrovirus (hereinafter, referred to as "RCR"). Above all, RCR production through homologous recombination has been a matter of grave concern.

All available retroviral vectors contain significantly long viral coding sequences. Since these sequences are also present in the genome of packaging cells from which the packaged vectors are released, it has been suggested that homologous recombination may occur between the same nucleotide sequence in the packaging genome and the vector, resulting in the production of RCR, which was reported by Miller et al. (Miller et al., Human Gene Ther., 1: 5, 1990).

Two types of MLV-based retroviral vectors, LN series vectors and MFG vector, have been most frequently used for gene therapy (Miller and Roseman, Biotechniques, 7:980-990, 1989; Dranoff et al., Proc. Nat'l. Acad. Sci. USA 90: 3539-3543, 1993). While the expression of a foreign gene in LN series vectors is controlled by a heterologous internal promoter or by LTR, transcription level in MFG vector is under the control of LTR, and the foreign gene is expressed as a

form of either genomic RNA or spliced subgenomic RNA. The LN series vectors, that are often considered the first generation retroviral vectors, contain the 420-bp *gag* coding sequence. Although this region has been thought to play an important role in viral packaging, it has been disclosed that *gag* region can be deleted without any significant effect on the viral packaging and titer under some conditions (Kim et al., J. Virol., 71: 994-1004, 1998).

Compared with LN series vectors, MFG vector is known to drive more stable and higher levels of gene expression, and produce higher viral titers in most of human- or mouse-derived cell lines (Byun et al., Gene Ther., 3: 780-788, 1996). However, MFG vector contains even more viral coding sequences, 420-bp for *gag*, 377-bp for *pol*, and 99-bp for *env*, raising the possibility of even higher frequency of producing RCR than the LN series vectors.

To overcome these disadvantages associated with conventional retroviral vectors, we, the inventors of this invention, constructed a retroviral vector (KOREA PATENT APPLICATION NO: 97-48095), which had several features as follows:

- transcripts of the cloned gene was effectively spliced, producing higher expression levels of the gene,

  gag and *env* sequences were completely deleted

without a loss of viral titer,

- IRES was used for the simultaneous expression of two or more genes in a vector,
- multicloning site was inserted into the vector to facilitate the cloning of foreign gene

Since *gag* and *env* sequences were deleted from the retroviral vectors, the safety of the vectors was increased when compared with those of other retroviral vectors. However, this vector still contains the 377-bp *pol* coding sequence that harbors the splicing acceptor sequence as well as its downstream sequence containing the 284-bp leader (transcribed but untranslated) sequence for *env*, because the deletion of *pol* sequence would lead to abnormal or inefficient splicing. Since the 377-bp for *pol* sequence is also present in the genome of packaging lines, the possibility of homologous recombination resulting in RCR production still remains in the vector.

To develop novel retroviral vectors with elevated efficiency of gene expression as well as enhanced safety, we have constructed the retroviral vectors that do not contain any viral coding sequence. This invention is performed by constructing retroviral vectors, where MLV-derived *pol* gene is completely deleted, excluding the possibility of RCR production through homologous recombination in packaging cell

line; where a heterologous intron, splicing acceptor, and/or non-coding sequence are/is inserted into the upstream position of cloning site for a foreign gene, maximizing the efficiency of gene expression; where either 5′ LTR or human cytomegalovirus (hereinafter, referred to as "HCMV") major immediate early promoter (MIEP: the promoter of *ie1* gene) is employed as *cis*-element for the regulation of foreign gene expression; and where either a heterologous internal promoter or an IRES is inserted in the the downstream position of the cloning site, allowing the simultaneous expression of two or more foreign genes.

SUMMARY OF THE INVENTION

It is an object of this invention to provide safe retroviral vectors where foreign gene(s) is/are expressed in higher levels and thus can be efficiently used for gene therapy.

In accordance with the present invention, the foregoing object is readily obtained.

This invention provides MLV-based retroviral vectors wherein the MLV-coding *gag*, *env* and *pol* sequences are completely deleted.

In one aspect, the MLV-based retroviral vectors of this invention may contain a heterologous intron and/or

a heterologous splicing acceptor which are/is inserted into the upstream position of cloning site for a foreign gene.

In another aspect, the MLV-based retroviral vectors of this invention may contain a heterologous non-coding sequence inserted into the upstream position of cloning site for a foreign gene.

In further aspect, the full-length U3 sequence (-419 to -1 bp) of MLV 5′ LTR may be replaced with a heterologous promoter in the MLV-based retroviral vectors of this invention.

In still further aspect, the MLV-based retroviral vectors of this invention may contain a heterologous promoter in the downstream position of cloning site for a foreign gene.

This invention also provides the *E. coli* strains transformed with the MLV-based retroviral vectors of this invention.

Further features of the present invention will appear hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG 1 represents the procedure where *pol*-coding region is deleted from MON vector to construct ΔSA vector, and then the CAT gene is inserted into the ΔSA vector to produce ΔSA-CAT vector,

FIG 2 schematically represents the structures of retroviral vectors containing the intron and/or exon of HCMV *ie1 (UL123) gene*,

FIG 3a represents the procedure where a SV40 minimal promoter-neo cassette is substituted for an IRES-neo cassette to obtain MSN vector, and then SN-3LTR vector is constructed through the insertion of the fragment containing SV40 minimal promoter and MLV 3' LTR into pUC18,

FIG 3b represents the procedure where a chimeric LTR containing HCMV major immediate early promoter is inserted into the SN-3LTR vector,

FIG 4a represents the procedure where a DNA fragment containing the exon 1, intron A and partial exon 2 of HCMV *ie1 (UL123) gene* is inserted into pCM vector to construct DON1.2 vector, and then the bacterial CAT gene is inserted into the DON1.2 to produce DON1.2-CAT,

FIG 4b represents the procedure where a DNA fragment containing the partial intron A and partial exon 2 of HCMV ie1 (UL123) gene is inserted into pCM vector to construct DON2 vector, and then the bacterial CAT gene is inserted into the DON2 to produce DON2-CAT,

FIG 4c represents the procedure where a splicing acceptor of mouse immunoglobulin gene and the exon 1 of HCMV ie1 (UL123) gene are inserted into pCM vector to construct DONSA1 vector, and then the bacterial CAT

gene is inserted into the DONSA1 to produce DONSA1-CAT,

FIG 5 schematically represents the structures of retroviral vectors containing the introns and exons of human genes,

FIG 6 represents the procedure where a DNA fragment containing MLV 5′ and 3′ LTR is prepared and inserted into pUC18 to give p53LTR vector, and then MIN vector is constructed through the insertion of the IRES-neo cassette isolated from pCBIN into the p53LTR.

FIG 7a represents the procedure of MIN-AI construction, wherein a genomic PCR product containing human β-actin gene is obtained, and then a DNA fragment containing the partial intron 1, splicing acceptor and partial exon 2 of human β-actin gene is inserted into the MIN vector.

FIG 7b represents the procedure of MIN-EI construction, wherein a genomic PCR product containing human EF1α gene is obtained, and then a DNA fragment containing the partial intron 1, splicing acceptor and partial exon 2 of human EF1α gene is inserted into the MIN vector.

FIG 7c represents the procedure of MIN-GI construction, wherein a DNA fragment containing the partial intron 1, splicing acceptor and partial exon 2 of human GAPDH gene is obtained through genomic PCR and then inserted into the MIN vector.

FIG 7d represents the procedure of MIN-2

construction, wherein a DNA fragment containing the partial intron A, splicing acceptor and partial exon 2 of HCMV ie1 (UL123) gene is inserted into the MIN vector.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention provides both safe and efficient retroviral vectors that are derived from MLV-based vectors, specifically from MON or MIN. In the vectors of this invention, retroviral genes (*gag*, *env* and *pol* coding sequences) are completely deleted; a heterologous intron, splicing acceptor, and/or non-coding sequence are/is inserted into the upstream position of cloning site for a foreign gene; a strong heterologous promoter is contained in 5′ LTR; and a heterologous promoter or an IRES is positioned in the downstream position of the cloning site.

Particularly, in the vectors of this invention, since *pol* coding sequence containing a splicing acceptor is completely deleted, the possibility of homologous recombination, which has been a disadvantage of the conventional retroviral vectors, can be excluded.

Deleting *pol* sequence leads to the reduction of gene expression level. To restore the reduced expression efficiency and virus titer, various retroviral vectors are provided in this invention, as

follows:

- This invention provides retroviral vectors where a heterologous intron and/or splicing acceptor are/is inserted into the upstream position of cloning site for a foreign gene in order to complement the deleted splicing acceptor that overlaps with the 3′ portion of *pol* coding sequence. All the introns and/or splicing acceptors of known viral or cellular genes may be used for this purpose, preferably the introns and/or splicing acceptors of HCMV ie1 (UL123) gene, elongation factor 1α (hereinafter, referred to as "EF1α") gene, glyceraldehyde 3-phosphate dehydrogenase (hereinafter, referred to as "GAPDH") gene, β-actin gene etc.
- This invention also provides retroviral vectors where a heterologous non-coding sequence is inserted in the upstream position of the cloning site. The non-coding sequence is used to promote translational efficiency, and defined as a transcribed DNA sequence that is not translated to a protein, including intron and untranslated exon. Preferably, the non-coding sequence is selectred from the group comprising the non-coding sequences of HCMV ie1 (UL123) gene, EF1α gene, GAPDH gene, and β-actin gene. The insertion of heterologous non-coding

sequence enables both the efficient splicing of foreign gene transcript and the effective translation of subgenomic RNA.

- In addition, this invention provides retroviral vectors where a heterologous internal promoter or IRES is harbored in the downstream position of the cloning site.
- Finally, this invention provides retroviral vectors where either a full-length U3 sequence of 5′ LTR or a strong heterologous promoter is contained.

Hereinafter, the present invention is described in detail.

1. The deletion of *pol* coding sequence: ΔSA construction

To develop retroviral vectors with improved safety, that is, retroviral vectors without the homologous recombination activity, *pol* coding sequence including a splicing acceptor was deleted from MON vector (KOREA PATENT APPLICATION NO: 97-48095), constructing a deletion mutant vector, ΔSA. In addition, ΔSA-CAT vector was constructed through the insertion of a bacterial CAT (; chloramphenicol acetyltransferase) as a reporter gene (see FIG 1). Subsequently, the cell lines transfected or transduced with the ΔSA-CAT were

prepared and brought to CAT assay. The CAT assay disclosed that the viral titer of the deletion mutant was comparable to that of parental vector MON-CAT but the gene expression was reduced (see Table 1). These results proposed that *pol* gene containing splicing acceptor be involved in the regulation of a foreign gene expression in a vector.

As expected from the results, the expression level of a foreign gene will be elevated if a heterologous splicing acceptor and/or intron are/is inserted into the upstream position of cloning site for a foreign gene so as to complement the splicing site deleted concommitantly with the *pol* gene. However, if the splicing takes place too much, the ratio of subgenomic RNA to genomic RNA will increase, leading to the decrease of viral titers in spite of the high levels of gene expression.

To construct the ideal retroviral vectors, therefore, the vectors should be designed so that the rates of transcription, splicing and translation may be balanced in transfected or transduced cell line. In other words, preferable are the vectors where a foreign gene is transcribed abundantly and the amount of genomic RNA is balanced with the amount of subgenomic RNA, so that genomic RNA may be produced enough to be packaged into viral particles and that subgenomic RNA may be translated into the plenty of protein encoded by

the foreign gene. In this invention, retroviral vectors are provided, where various non-coding sequences originated from viral or cellular genes are inserted into the deletion mutant vector, and then it is investigated whether this insertion has an effect on the viral titers and gene expression levels.

2. The insertion of a heterologous DNA sequence enhancing splicing and trnaslational efficiency: DON1.2, DON2 and DONSA1 construction

In order to keep the balance of splicing rate and gene expression rate as well as to elevate the overall yield of viral RNA, retroviral vectors were constructed where the U3 region of MLV 5' LTR was replaced with a strong promoter, HCMV major immediate early promoter, and where a heterologous non-coding sequence was inserted.

In a preferred embodiment, the untranslated exon and/or intron of HCMV ie1 (UL123) gene were/was employed as the heterologous non-coding sequence which was inserted into the upstream position of the cloning site of the vector (see FIG 2). It has been known that the exon and/or intron sequences of HCMV ie1 (UL123) gene enhance the translation efficiency when inserted into an expression vector.

Particularly, pCM vector was prepared as follows: all of retroviral coding sequences were deleted; the

full-length U3 sequence of 5' LTR was replaced with HCMV major immediate early promoter, with the MLV-derived 3'LTR kept up; and SV40 promoter-neo cassette was employed as a heterologous internal promoter. Subsequently, DON1.2, DON2 and DONSA1 vectors were constructed through inserting the exon 1, intron A, and/or exon 2 of HCMV ie1 (UL123) gene into the pCM vector (see FIG 3a, FIG 3b, and FIG 4a to 4c). Then, the DON1.2-CAT, DON2-CAT, and DONSA1-CAT vectors were prepared through the insertion of CAT reporter gene. Cell lines transfected or tansduced with these vectors were brought to CAT assay. From the result of CAT assay, it was confirmed that all three vectors showed much higher efficiencies of splicing and gene expression than the control vector L-CAT-SN (see Table 2).

3. The insertion of cellular DNA sequences which can promote splicing and gene expression: MIN-AI, MIN-EI, and MIN-GI construction

In another preferred embodiment, retroviral vectors were further devised so that the balance of splicing rate and gene expression rate might lead to higher efficiencies both of viral titers and of gene expression. In these vectors, the exon and/or intron sequences of human genes were inserted into the upstream position of cloning site for foreign gene in

the retroviral vectors (see FIG 5).

To construct these vectors, MIN vector was prepared where all of retroviral coding region was deleted; MLV-derived 5′ and 3′ LTR were maintained; and EMCV IRES-neo cassette was employed as a heterologous internal promoter. Especially, MIN vector was prepared through following steps: the amplification of a DNA fragment containing MLV 5′ and 3′ LTR region, the insertion of this fragment into pUC18 vector, and the insertion of IRES-neo cassette into the recombinant vector (see FIG 6). A DNA fragment containing the partial intron and partial exon 2 of human β-actin, EF1α, or GAPDH gene, was inserted into the upstream position of the cloning site of MIN vector, constructing MIN-AI, MIN-EI, or MIN-GI vector, respectively (see FIG 7a to 7c).

To compare the vector using cellular gene as a splicing acceptor (e.g. MIN-AI, etc.) with one using a viral gene (e.g. DON1.2, etc.), another MLV-based vector MIN-2 was prepared. Specifically, DNA sequence containing the partial intron A and partial exon 2 of HCMV iel (UL123) gene is inserted into MIN vector (see FIG 7d). This insert is identical to that of DON2 vector.

Then, MIN-CAT, MIN-AICAT, MIN-EICAT, MIN-GICAT and MIN-2CAT vectors were prepared through the insertion of CAT reporter gene into the corresponding vector. Cell

lines transfected or transduced with these vectors were brought to CAT assay. The result of the CAT assay disclosed that the viral titers of all the vectors of this invention are similar to those of control vectors LXSN and MFG, while the gene expression levels in cell lines transfected or transduced with MIN-2 and MIN-EI are much higher than cell lines containing the control vectors (see Table 3).

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: The deletion of *pol* gene from MON vector

(1-1) ΔSA construction

To prepare the 5′ LTR downstream sequence lacking *pol* coding sequence, PCR (; Polymerase Chain Reaction) was performed, wherein MON vector (KOREA PATENT APPLICATION NO: 97-48095) was employed as a template and two single-stranded synthetic oligonucleotides

described by SEQ ID NO: 1 (HHIR primer) and NO: 2 (R523Bam primer) were as primers. The amplified DNA fragments corresponded to the nucleotide sequence from MLV 5′ LTR to +523 bp (see FIG 1). In this case, +1 represents the transcription start site of MLV genome, and the sequence from +212 to +523 bp is a non-coding sequence that is required to package genomic RNA.

The PCR product was subcloned into pCRII (Invitrogen, CA, USA), and then the *Hin*dIII-*Bam*HI fragment was prepared from the resulting vector. A partial *Hin*dIII-*Bam*HI fragment of MON vector was replaced with the *Hin*dIII-*Bam*HI fragment of PCR product, constructing ΔSA vector. This ΔSA vector is used as an elementary backbone of retroviral vectors which contain MLV 5′ and 3′ LTR, and 5′ non-coding sequence comprising a splicing acceptor, IRES-neo cassette and polypurine track, with all of retroviral coding sequences deleted (see FIG 1).

(1-2) ΔSA-CAT construction

To investigate the effect of the above genetic manipulation on the foreign gene expression and on the packaging of retroviral genomic RNA, a bacterial CAT gene was employed as a reporter gene.

CAT gene was obtained through PCR, in which pCAT3-basic vector (Promega, WI, USA) was used as a template and two synthetic oligonucleotides described by SEQ ID

NO: 3 (CATATGN primer) and NO: 4 (CATSTOP primer) were as primers. The PCR product was inserted into the pCRII (Invitrogen, CA, USA), and the *Bam*HI fragment of the resulting vector, containing CAT gene, was introduced into pC3.1 (Invitrogen, CA, USA) to prepare pC3.1-CAT. ΔSA-CAT vector was constructed through the insertion of CAT gene (Klenow-treated *Xba*I-*Hin*dIII fragment of pC3.1-CAT) into the *Hpa*I site of ΔSA vector (see FIG 1).

(1-3) CAT assay

ΔSA-CAT and control vector (MON-CAT), together with Gag-Pol and Env packaging vectors, were transfected to 293T cells (DuBridge et al., Mol. Cell. Biol., 7: 379-387, 1987). After the transfected cells were cultured for 48 hours, cytosolic proteins were extracted to measure CAT activity, an indicator of foreign gene expression level. Meanwhile, cell-free viral supernatants, obtained by filtrating the cultured medium with 0.45-μm filter, were used to transduce NIH3T3 cells (ATCC CRL 1658). The level of CAT activity was determined using the protein extract 48 hours after the transduction.

CAT assay was performed as follows: first, cells were harvested and washed with 1 ml of PBS (phosphate-buffered saline), and then resuspended in 0.25 M Tris buffer (pH 7.5). The cells were lyzed through

repeating freezing (in dry ice)- thawing (in 37°C water bath) cycle 6 times. After the resulting cell extract was heated at 60°C for 7 minutes to inactivate deacetylase, it was centrifuged at 12,000 rpm for 10 minutes and the supernatant was rescued. The protein level in the extract was quantified according to Bradford's method. The normalized extract was mixed with 1 μl of ^{14}C-chloramphenicol (60 mCi/mmole, 0.1 mCi/ml), 2 μl of acetyl-coenzyme A (40 mM), and appropriate volume of 0.25 M Tris (pH 7.5). This reaction mixture was incubated at 37°C for appropriate reaction time. After the reaction, chloramphenicol was extracted with ethyl acetate and concentrated under reduced pressure. The pellet was resuspended in 15 μl of ethyl acetate and loaded onto thin layer chromatography (TLC) plate. After TLC was performed using TLC developing solvent (95% choloroform, 5% methanol), TLC plate was dried and then exposed to X-ray film or brought to phosphoimage analyzer, so that the acetylation level of chloramphenicol may be measured. CAT activity in a sample could be calculated from the radioactivity ratio of acetylated chloramphenicol to total chloramphenicol.

Table 1 shows that CAT activity in 293T cell line transfected with ΔSA-CAT lacking *pol* gene was lower than in control line transfected with MON-CAT. This suggests that *pol* coding sequence be involved in the

regulation of gene expression. In addition, CAT activity in transduced line showed the similar pattern to that in transfected line, implying that the packaging efficiency is related to the gene expression efficiency.

Table 1. The effect of *pol* deletion on gene expression level

Vector	Cell line	
	293T cells	NIH3T3 cells
MON-CAT	1.0	1.0
ΔSA-CAT	0.5 ± 0.1	0.4 ± 0.1

Example 2: The insertion of promoter and exon and/or intron of HCMV ie1 (UL123) gene

In order to keep the balance of splicing rate and translation rate as well as to enhance the overall yield of viral RNA, MON-derived retroviral vectors comprising the following three vectors were constructed:

- DON1.2 vector where the full-length U3 sequence of MLV 5′ LTR is replaced with HCMV major immediate early promoter; and MLV splicing acceptor is replaced with a DNA fragment

containing the exon 1, intron A and partial exon 2 (which ends just before start codon) of HCMV ie1 (UL123) gene,

- DON2 vector where the full-length U3 sequence of MLV 5′ LTR is replaced with HCMV major immediate early promoter; and MLV splicing acceptor is replaced with DNA fragment containing the partial intron A and partial exon 2 of HCMV ie1 (UL123) gene, and
- DONSA1 vector where the full-length U3 sequence of MLV 5′ LTR is replaced with HCMV major immediate early promoter; and MLV splicing acceptor is replaced with a DNA fragment containing the splicing acceptor of mouse immunoglobulin gene and the exon 1 of HCMV ie1 (UL123) gene

The detailed method of constructing these vectors is described as follows (see FIG 3a, FIG 3b, and FIG 4a to 4c).

(2-1) SN-3LTR construction

In three variant vectors, SV40 promoter-neo cassette is inserted into the downstream position of cloning site for a foreign gene (see FIG 2). To construct these variant vectors, a vector containing SV40 promoter-neo cassette was prepared as follows (see FIG 3a and 3b).

To prepare a vector where neo gene is expressed under the control of SV40 promoter, SV40 promoter-neo cassette was produced through PCR. In the PCR, pC3.1 (Invitrogen, CA, USA) was employed as a template, and two synthetic oligonucleotides described by SEQ ID NO: 5 (SV40-5 primer) and NO: 6 (Neo-3 primer) were as PCR primers.

After the SV40 promoter-neo cassette was subcloned in pCRII (Invitrogen, CA, USA), the *Bam*HI-*Xho*I fragment of the resulting vector was inserted into the *Bam*HI/*Xho*I site of MON. The resulting vector, MSN was digested with *Bam*HI and *Eco*RI restriction enzymes, and the *Bam*HI-*Eco*RI fragment containing SV40 promoter-neo cassette and 3′ LTR was ligated with the *Bam*HI-*Eco*RI fragment of pUC18 so as to construct SN-3LTR vector (see FIG 3a).

(2-2) pCM construction

PCR was performed in order to prepare retroviral vector where 5′ LTR was replaced with a strong heterologous promoter. Employed as a PCR template was a retroviral vector MCC-CAT (KOREA PATENT APPLICATION NO: 97-48095) containing chimeric LTR in which full-length U3 sequence (-419 to -1 bp) of MLV 5′ LTR was replaced with the full-length HCMV major immediate early promoter. PCR primers were two synthetic oligonucleotides, described by SEQ ID NO: 1 (HHIR

primer) and NO: 2 (R523Bam primer). The PCR products contained the DNA sequence from chimeric 5′ LTR to +523 bp and subcloned in pCRII (Invitrogen, CA, USA). The *Hin*dIII-*Bam*HI fragment of the subcloned sequence was inserted into the *Hin*dIII-*Bam*HI site of SN-3LTR vector (obtained in Example 2-1) to prepare pCM vector (see FIG. 3b). In the pCM, all of the retroviral coding sequences were deleted just like ΔSA vector of Example 1-1, while 5′ LTR and IRES-neo cassette of ΔSA vector were replaced with chimeric LTR and SV40 promoter-neo cassette, respectively. The pCM vector was employed as a starting material for DON1.2, DON2 and DONSA1 vector, as shown in Example 2-3 to 2-5.

(2-3) DON1.2 and DON1.2-CAT construction

To obtain DNA fragment containing the exon 1, intron A and exon 2 (to start codon) of HCMV ie1 (UL123) gene, PCR was performed. Template in the PCR was pEQ276 vector (Biegalke et al., Virology, 183: 381-385, 1991) containing DNA sequence from HCMV major immediate early promoter to the exon 5. Two PCR primers were described by SEQ ID NO: 7 (RI5 primer, hybridized with the exon 1) and NO: 8 (CMVexon2.3 primer, hybridized with the exon 2), respectively. 1-kb DNA fragment was amplified in the PCR and contained the exon 1, intron A, and partial exon 2 (which ends just before start codon) of HCMV ie1 (UL123) gene.

The PCR product was inserted into pZero-blunt vector (Invitrogen, CA, USA) to prepare pZero1.2 vector. Then, *Eco*RI fragment of pZero1.2 was treated with Klenow enzyme to make blunt ends. The pCM vector obtained in Example 2-2 was digested with *Bam*HI enzyme and treated with Klenow enzyme. Two DNA fragments with blunt ends were ligated to construct DON1.2 vector. Additionally, DON1.2-CAT vector was constructed through the insertion of a CAT gene into the Klenow-treated *Hin*dIII fragment of DON1.2 (see FIG 4a).

(2-4) DON2 and DON2-CAT construction

The *Hpa*I-*Eco*RI fragment of pZero1.2 of Example 2-3 was prepared, which contained 112-bp 3′ region of the intron A and 5′ region of exon 2 (from +837 to +964, just before start codon). Then, this DNA fragment was treated with Klenow enzyme to make blunt ends. On the other hand, the pCM vector obtained in Example 2-2 was digested with *Bam*HI enzyme and treated with Klenow enzyme. The above two DNA fragments with blunt ends were ligated to construct DON2 vector. Additionally, DON2-CAT vector was constructed through the insertion of a CAT gene into the Klenow-treated *Hin*dIII fragment of DON2 (see FIG 4b).

(2-5) DONSA1 and DONSA1-CAT construction

To prepare the splicing acceptor of mouse

immunoglobulin gene, single-stranded oligonucleotides were synthesized, which were described by SEQ ID NO: 9 (SA Top oligomer) and NO: 10 (SA bottom oligomer), respectively. Annealing reaction of the two oligomers produced splicing acceptor fragments with cohesive ends. pGEM4-SA vector was prepared through the insertion of the fragment into the *Bam*HI site of pGEM4 vector (Promega, WI, USA) (see FIG 4c).

To amplify the exon 1 of HCMV ie1 (UL123) gene, PCR was performed, wherein the pEQ276 vector of Example 2-3 was employed as a template and two synthetic oligonucleotides described by SEQ ID NO: 7 (RI5 primer) and NO: 11 (exon 13 primer), respectively, as primers.

The amplified exon 1 fragment was subcloned in the *Eco*RI site of pZero-blunt vector (Invitrogen, CA, USA), producing pZero-exon1 vector. Then, *Eco*RI fragment of pZero-exon1 was subcloned in the *Eco*RI site of the pGEM4-SA to prepare pGEM-SA-exon1 vector.

The *Xba*I-*Bam*HI fragment of pGEM4-SA-exon1 was treated with Klenow to make blunt ends, and then ligated with the Klenow-treated *Bam*HI fragment of pCM (obtained in Example 2-2), constructing DONSA1 vector.

Additionally, DONSA1-CAT vector was constructed through the insertion of CAT gene into the *Hpa*I site of DONSA1 (see FIG 4c).

(2-6) CAT assay

DON1.2-CAT, DON2-CAT, DONSA1-CAT and L-CAT-SN vector were transfected to packaging line FlyA13 (Cosset et al., J. Virol., 69: 7430-7436, 1995). After the transfected lines were cultured for 48 hours, cell-free viral supernatants were prepared to transduce NIH3T3 cells. The transduced lines were cultured for 48 hours. CAT activities in the transfected or transduced lines were measured according to the method of Example 1-3, thereby analyzing the relative gene expression levels.

As shown in Table 2, markedly higher level of CAT activity was observed in FlyA13 line transfected with DON1.2-CAT or DON2-CAT than in a control line (transfected with L-CAT-SN). In addition, NIH3T3 cells transduced stably with DON1.2-CAT or DON2-CAT vector showed 5- to 10-fold higher CAT activity than control cells did. In case of cell line transfected or transduced with DONSA1-CAT, a little higher CAT activity was observed than in control. These results suggested that splicing efficiency and gene expression efficiency can be elevated if a heterologous non-coding sequence involved in splicing is inserted into the upstream position of foreign gene in an expression vector.

E. coli strains transformed with DON2 and DONSA1 vector were designated TOP10-DON2 and Top10-DONSA1, respectively. They were deposited in Korean Culture

Center of Microorganism on June 5, 1998 (accession NO: KCCM-10128, KCCM-10127, respectively).

Table 2. The effect of the heterologous sequence insertion on the gene expression level

Vector	Cell line	
	FlyA13	NIH3T3
L-CAT-SN	1.0	1.0
DON1.2-CAT	10.1 ± 1.5	7.7 ± 1.6
DON2-CAT	12.5 ± 2.1	8.0 ± 2.0
DONSA1-CAT	5.0 ± 2.0	1.8 ± 1.2

Example 3: The insertion of the intron and/or exon of human gene

In this Example, MIN-derived retroviral vectors, MIN-AI, MIN-EI, MIN-GI and MIN-2 were constructed, so that the rates of transcription, splicing and translation of a foreign gene might be balanced in eukaryotic cells. None of viral sequences are contained in MIN vector as well as in ΔSA vector, but MIN contains IRES-neo cassette instead of SV promoter-neo cassette (see FIG 5). The features of the above four vectors are as follows:

A DNA fragment containing the intron, splicing

acceptor and partial exon 2 of human β-actin gene was inserted into the upstream position of foreign gene in MIN-AI vector.

- A DNA fragment containing the intron, splicing acceptor and partial exon 2 of human EF1α gene was inserted into the upstream position of foreign gene in MIN-EI vector.
- A DNA fragment containing the intron, splicing acceptor and partial exon 2 of human GAPDH gene was inserted into the upstream position of foreign gene in MIN-GI vector.

Besides these constructs, a vector was prepared, where a heterologous, viral sequence was inserted. Especially, MIN-2 vector was prepared where a DNA fragment containing the intron, splicing acceptor and partial exon 2 of HCMV ie1 (UL123) gene was inserted into the upstream position of cloning site of the MIN vector.

MIN and MIN-derived MIN-AI, MIN-EI, MIN-GI and MIN-2 vectors were constructed as follows.

(3-1) MIN construction

To obtain MLV 3′ LTR region, PCR was performed, in which pMLV (Shinnick et al., Nature, 293: 543-548, 1981) was employed as a template and two synthetic oligonucleotides described by SEQ ID NO: 12 (3LTR5 primer) and NO: 13 (3LTR3 primer) were as primers.

Amplified PCR product contained the 3′ untranslated region, the polypurine track, and the 3′ LTR of MLV genome. PCR product subcloned in pCRII vector (Invitrogen, CA, USA) was digested with *Bam*HI and *Eco*RI enzymes, and the resulting fragment was inserted into the *Bam*HI-*Eco*RI site of pUC18 to prepare p3LTR vector (see FIG 6).

On the other hand, to obtain non-coding sequence containing retroviral 5′ LTR and splicing donor, PCR was performed, where pMLV was employed as a template and two synthetic oligonucleotides described by SEQ ID NO: 1 (HHIR primer) and NO: 14 (5LTR3 primer) were as primers. Amplified PCR product contained nucleotide sequence from 5′ LTR to +623 bp (just before *gag* coding sequence) of MLV genome. After PCR product was subcloned in pCRII vector, the *Hin*dIII-*Bam*HI fragment of the vector was inserted into the *Hin*dIII-*Bam*HI site of p3LTR to prepare p53LTR vector (see FIG 6).

Finally, IRES/neo cassette were isolated from pCBIN (KOREA PATENT APPLICATION NO: 97-48095) through *Bam*HI-*Xho*I digestion, and then inserted into *Bam*HI-*Xho*I site of the p53LTR vector to construct MIN vector (see FIG 6).

MIN-CAT vector was also constructed through the insertion of a CAT gene into the *Bam*HI site of MIN vector so as to analyze the expression efficiency of MIN vector.

(3-2) MIN-AI construction

To prepare human nucleotide sequences that would be inserted into MIN vector, genomic DNA was extracted from human cells. First, peripheral blood mononuclear cells were separated from human blood by Ficoll-paque gradient centrifugation. After washed once or twice and gathered again, the cells were lysed with TES (10 mM Tris-Cl (pH 7.0), 10 mM EDTA, 0.7 % SDS). Proteinase K (400 μg/ml) was added to the cell lysate, and the lysate was incubated at 50-55°C for 1-2 hours, followed by phenol/chloroform extraction and ethanol precipitation.

The isolated genomic DNA was employed as a template in PCR, which amplified DNA fragment containing the promoter, exon 1, intron and partial exon 2 of human β-actin gene. The PCR primers were two synthetic oligonucleotides described by SEQ ID NO: 15 (beta-actin 5 primer) and NO: 16 (beta-actin 3 primer). The PCR product was subcloned in pCRII vector, and then the *Mlu*I-*Nhe*I fragment of the vector was inserted into the *Mlu*I-*Nhe*I site of pC3.1 vector (Invitrogen, CA, USA) to prepare pβactin.

The Klenow-treated *Bgl*I-*Bam*HI fragment of pβactin (corresponding to +717 ~ +849 of human β-actin gene) was inserted into the T4-polymerase-treated *Apa*I/*Bam*HI site of MIN vector (see FIG 7a). The resulting vector

was designated MIN-AI.

In addition, MIN-AICAT vector was constructed through the insertion of a CAT gene into the *Bam*HI site of MIN-AI vector so as to analyze the expression efficiency of MIN-AI.

(3-3) MIN-EI construction

To obtain the non-coding sequence of human EF1α gene, genomic PCR was performed. The genomic DNA isolated in Example 3-2 was employed as a template in the PCR, and two synthetic oligonucleotides described by SEQ ID NO: 17 (EF1α5 primer) and NO: 18 (EF1α3 primer) were as PCR primers. The PCR product contained the promoter, exon 1, intron and partial exon 2 of human EF1α gene. The PCR product was subcloned in pCRII vector, and then the *Mlu*I-*Nhe*I fragment of the vector was inserted into the *Mlu*I-*Nhe*I site of pC3.1 vector (Invitrogen, CA, USA) to prepare pEF1α.

The Klenow-treated *Xho*I-*Bam*HI fragment of pEF1α (corresponding to +772 ~ +1008 of human EF1α gene) was inserted into the T4-polymerase-treated *Apa*I-*Bam*HI site of MIN vector (see FIG 7b). The resulting vector was designated MIN-EI.

In addition, MIN-EICAT vector was constructed through the insertion of CAT cassette into the *Bam*HI site of MIN-EI vector in order to analyze the expression efficiency of MIN-EI vector. The MIN-EICAT

was introduced into *E. coli* strain Top10, and the *E. coli* transformant was designated MIN-EICAT(Top10) and deposited in Korean Culture Center of Microorganisms on June 2, 1999 (Accession NO: KCCM-10163).

(3-4) MIN-GI construction

To obtain the non-coding sequence of human GAPDH gene, genomic PCR was performed. The genomic DNA isolated in Example 3-2 was employed as a template in the PCR, and two synthetic oligonucleotides described by SEQ ID NO: 19 (Gint5 primer) and NO: 20 (Gint3 primer) were as PCR primers. The PCR product contained the partial intron and partial exon 2 of human GAPDH gene, corresponding to +185 ~ +317 of human GAPDH gene. The PCR product was subcloned in pCRII vector, and then the *Mlu*I-*Bam*HI fragment of the vector was inserted into the *Mlu*I-*Bam*HI site of MIN vector. The resulting vector was designated MIN-GI (see FIG 7c).

In addition, MIN-GICAT vector was constructed through the insertion of a CAT gene into the *Bam*HI site of MIN-GI vector in order to analyze the expression efficiency of MIN-GI vector.

(3-5) MIN-2 construction

The *Mlu*I-*Bam*HI fragment of DON2 (corresponding to +837 ~ +964 of HCMV *ie1* gene) was prepared, which contained the intron, splicing acceptor and partial

exon 2 of HCMV ie1 (UL123) gene. This *Mlu*I-*Bam*HI fragment was inserted into the *Mlu*I-*Bam*HI site of MIN vector (see FIG 7d). The resulting vector was designated MIN-2.

In addition, MIN-2CAT vector was constructed through the insertion of CAT gene into the *Bam*HI site of MIN-2 so as to analyze the expression efficiency of MIN-2 vector. The MIN-2CAT was introduced into *E. coli* strain Top10. The *E. coli* transformant was designated MIN-2CAT(Top10) and deposited in Korean Culture Center of Microorganisms on June 2, 1999 (Accession NO: KCCM-10164).

(3-6) CAT assay

To investigate the foreign gene expression efficiencies and the packaging capabilities of the above four vectors, the vectors were brought to CAT assay, wherein the CAT-inserted forms of well-known retroviral vectors, MFG and LXSN, were employed as control vectors (Miller et al., Biotechniques, 7: 980-990, 1989; Ohashi et al., Proc. Natl. Acal. Sci. USA, 89: 11332-11336, 1992). The packaging line Phoenix (Kinsella and Nolan, Hum. Gene. Ther. 7: 1405-1413) was transfected with these vectors and then cultured for 48 hours. Meanwhile, cell-free viral supernatants, obtained by filtrating the cultured medium with 0.45 µm filter, were used to transduce NIH3T3 cells (ATCC CRL

1658). The level of CAT activity was determined using the protein extract 2 days after transduction. CAT activity was measured in cell line transfected or transduced with each vector (see the columns "transiently transfected" and "transduced, transient" in Table 3) as well as in antibiotics-resistant cell population (see the column "transduced, stable" in Table 3). Additionally, CAT activity was measured in the stable cell population subcultured for 4 weeks (see the column "transduced, stable (4 weeks)" in Table 3). The gene expression levels and the viral titers in transfected lines were determined from CAT activities that were measured in transfected or transduced line with each vector.

As shown in Table 3, all the retroviral vectors showed higher CAT activities than MIN vector, except for LXSN vector. However, the CAT activities were varied, depending on the heterologous sequence inserted. Especially, MIN-EI and MIN-2 produced strikingly high levels of gene expression. It was quite remarkable that the cells transduced stably with MIN-EI or MIN-2 produced far higher gene expression levels, even after the subculture for 4 weeks.

Table 3. The effect of heterologous sequences on the efficiency of retroviral vectors

Vector	Relative CAT activity				Relative viral titer
	Transiently transfected	Transduced			
		Transient	Stable	Stable (4 weeks)	
MIN-CAT	1.0	1.0	1.0	1.0	1.0
MIN-2CAT	3.8 ± 0.5	3.7 ± 0.5	4.5 ± 0.5	2.3 ± 0.5	0.9 ± 0.3
MIN-AICAT	0.9 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	0.9 ± 0.2	0.8 ± 0.2
MIN-EICAT	3.5 ± 0.4	3.3 ± 0.4	3.5 ± 0.4	2.7 ± 0.4	1.1 ± 0.2
MIN-GICAT	2.0 ± 0.5	2.4 ± 0.3	2.2 ± 0.4	1.5 ± 0.3	1.3 ± 0.2
MFG-CAT	1.0 ± 0.2	1.1 ± 0.3	0.8 ± 0.2	0.3 ± 0.1	1.0 ± 0.3
LXSN-CAT	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.5 ± 0.2

INDUSTRIAL APPLICABILITY

As disclosed and verified above, this invention provides retroviral vectors which have many advantages for gene therapy and so on. The vectors of this invention have following features:

1. Since all of retroviral coding sequences (*gag*, *env*, and *pol* sequences) are deleted, the possibility can be utterly excluded that replication-competent retrovirus is produced through homologous recombination.

2. Since a heterologous intron, splicing acceptor, and/or non-coding sequence are/is inserted into the upstream position of cloning site for a foreign gene, the foreign gene in the vectors can be expressed both stably and efficiently.

3. Since U3 region in 5′ LTR is replaced with a heterologous promoter that intensively induces transcription especially in human cells, the human cell-derived packaging lines transfected with the vectors can produce higher levels of RNA and thus show increased viral titers.

4. An IRES or a heterologous promoter is used simultaneously to express two or more foreign genes in the vectors. In this case, a minimal promoter may be employed as the inserted heterologous promoter, in order to diminish an interference of the heterologous internal promoter and to clone a foreign gene with larger size.

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

1/2

PCT

Original (for SUBMISSION) - printed on 24.06.1999 03:13:52 PM

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.84 (updated 01.06.1999)
0-2	International Application No	
0-3	Applicant's or agent's file reference	9FPO-05-10

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	27, 39
1-2	line	2, 24
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	Korean Culture Center of Microorganisms
1-3-2	Address of depositary institution	Department of Food Engineering College of Eng. Yonsei University Sodaemun-gu, Seoul 120-791, Korea
1-3-3	Date of deposit	05 June 1998 (05.06.1998)
1-3-4	Accession Number	KCCM 10127
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
2-1	page	27,39
2-2	line	2,26
2-3	Identification of Deposit	
2-3-1	Name of depositary institution	Korean Culture Center of Microorganisms
2-3-2	Address of depositary institution	Department of Food Engineering College of Eng. Yonsei University Sodaemun-gu, Seoul 120-791, Korea
2-3-3	Date of deposit	05 June 1998 (05.06.1998)
2-3-4	Accession Number	KCCM 10128
2-4	Additional Indications	NONE
2-5	Designated States for Which Indications are Made	all designated States
2-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE
3	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
3-1	page	32,40
3-2	line	4,1

PCT

Original (for SUBMISSION) - printed on 24.06.1999 03:13:52 PM

3-3	Identification of Deposit	
3-3-1	Name of depositary institution	Korean Culture Center of Microorganisms
3-3-2	Address of depositary institution	361-221, Yurim B/D, Honje-1-dong, Sudaemun-gu, Seoul, 120-091, Republic of Korea
3-3-3	Date of deposit	02 June 1999 (02.06.1999)
3-3-4	Accession Number	KCCM 10163
3-4	Additional Indications	NONE
3-5	Designated States for Which Indications are Made	all designated States
3-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE
4	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
4-1	page	33,40
4-2	line	12,6
4-3	Identification of Deposit	
4-3-1	Name of depositary institution	Korean Culture Center of Microorganisms
4-3-2	Address of depositary institution	361-221, Yurim B/D, Honje-1-dong, Sudaemun-gu, Seoul, 120-091, Republic of Korea
4-3-3	Date of deposit	02 June 1999 (02.06.1999)
4-3-4	Accession Number	KCCM 10164
4-4	Additional Indications	NONE
4-5	Designated States for Which Indications are Made	all designated States
4-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

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0-4	This form was received with the international application: (yes or no)	YES
0-4-1	Authorized officer	LEE, Seung Jong

FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the international Bureau on:	
0-5-1	Authorized officer	

LAST ADDITIONAL SHEET

What is claimed is

1. MLV-based retroviral vector, wherein the MLV (; murine leukemia virus)-coding *gag*, *env* and *pol* sequences are completely deleted.
2. The MLV-based retroviral vector of claim 1, which contains a heterologous intron and/or a heterologous splicing acceptor which are/is inserted into the upstream position of cloning site for a foreign gene.
3. The MLV-based retroviral vector of claim 2, wherein the heterologous intron and/or the heterologous splicing acceptor are/is selected from the group comprising the introns and/or splicing acceptors of HCMV (; human cytomegalovirus) iel (UL123) gene, EF1α (; elongation factor 1α) gene, GAPDH (; glyceraldehyde 3-phosphate dehydrogenase) gene, β-actin gene, and mouse immunoglobulin gene.
4. The MLV-based retroviral vector of claim 1, which contains a heterologous non-coding sequence inserted into the upstream position of cloning site for a foreign gene.
5. The MLV-based retroviral vector of claim 4, wherein the heterologous non-coding sequence is selected from the group comprising the non-coding

sequences of HCMV ie1 (UL123) gene, EF1α gene, GAPDH gene, and β-actin gene.

6. The MLV-based retroviral vector of claim 1, wherein the full-length U3 sequence (-419 to -1 bp) of MLV 5′ LTR is replaced with a heterologous promoter.

7. The MLV-based retroviral vector of claim 6, wherein the heterologous promoter is HCMV major immediate early promoter.

8. The MLV-based retroviral vector of claim 1, which contains a heterologous promoter inserted into the downstream position of cloning site for a foreign gene.

9. The MLV-based retroviral vector of claim 8, wherein the heterologous promoter is SV40 minimal promoter.

10. The MLV-based retroviral vector of claim 2, which is DONSA1 vector wherein the splicing acceptor of mouse immunoglobulin gene and the exon 1 of HCMV ie1 (UL123) gene are inserted into the upstream position of cloning site for the foreign gene; the full-length U3 sequence (-419 to -1 bp) of MLV 5′ LTR is replaced with HCMV major immediate early promoter; and SV40 minimal promoter is inserted into the downstream position of cloning site for the foreign gene.

11. The MLV-based retroviral vector of claim 2, which

is DON2 vector wherein the partial intron A (112-bp 3' fragment) and partial exon 2 (5' fragment covering from 5' end of exon 2 to the sequence just before start codon) of HCMV ie1 (UL123) gene are inserted into the upstream position of cloning site for the foreign gene; the full-length U3 sequence (-419 to -1 bp) of MLV 5' LTR is replaced with HCMV major immediate early promoter; and SV40 minimal promoter is inserted into the downstream position of cloning site for the foreign gene.

12. The MLV-based retroviral vector of claim 4, which is MIN-EI vector wherein a DNA fragment containing the partial intron and partial exon 2 (+772 to +1008 bp) of human EF1α gene is inserted into the upstream position of cloning site for the foreign gene.

13. The MLV-based retroviral vector of claim 4, which is MIN-2 vector wherein a DNA fragment containing the partial intron A and partial exon 2 (+837 to +964 bp) of HCMV ie1 (UL123) gene is inserted into the upstream position of cloning site for the foreign gene.

14. *E. coli* strain Top10-DONSA1 (Accession NO: KCCM-10127) transformed with DONSA1 vector of claim 10.

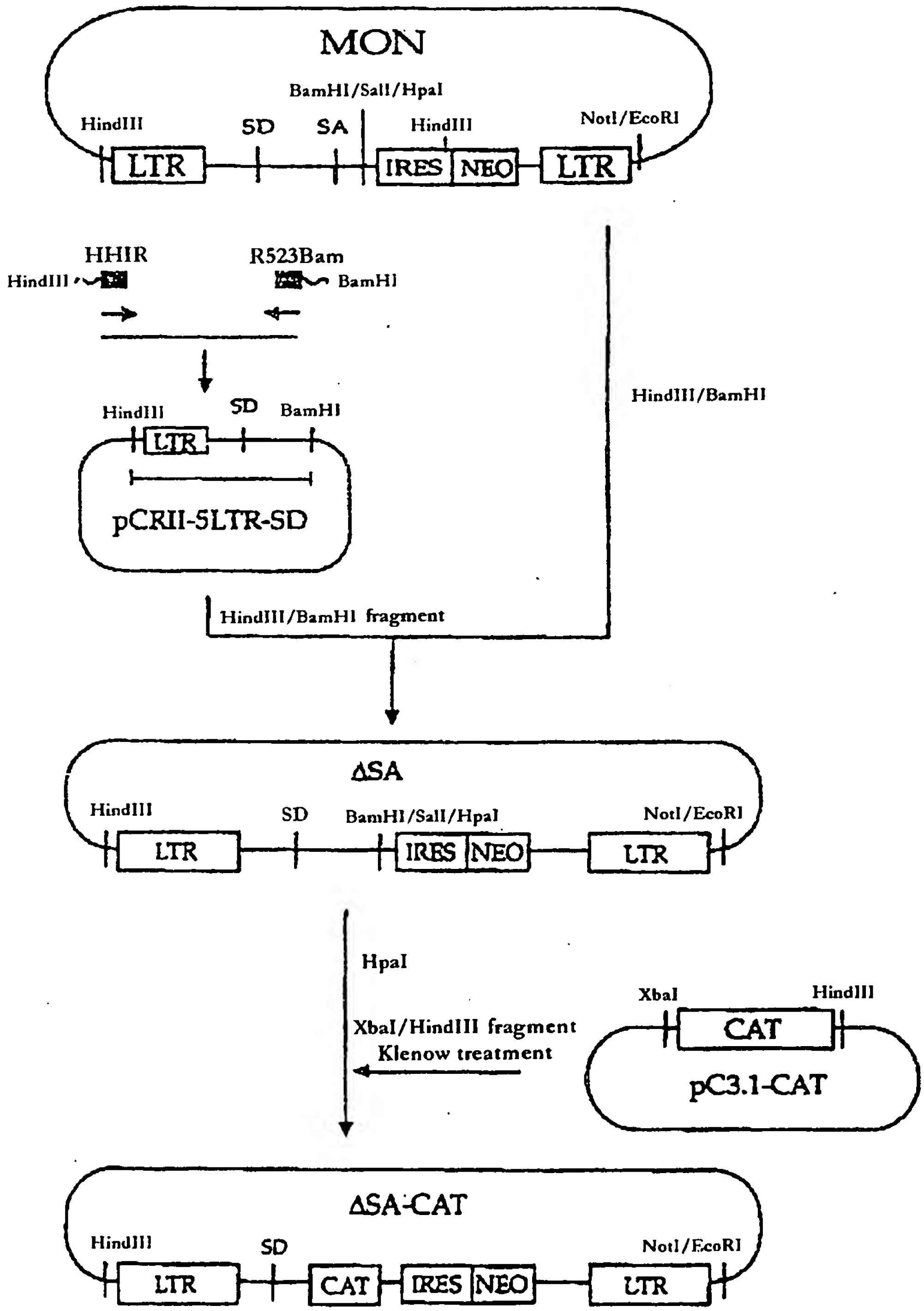
15. *E. coli* strain Top10-DON2 (Accession NO: KCCM-10128) transformed with DON2 vector of claim 11.

16. *E. coli* strain MIN-EICAT(Top10) (Accession NO:

KCCM-10163) transformed with MIN-EICAT vector which contains a bacterial CAT (; chloramphenicol acetyltransferase) gene in the cloning site of the vector of claim 12.

17. *E. coli* strain MIN-2CAT(Top10) (Accession NO: KCCM-10164) transformed with MIN-2CAT vector which contains a bacterial CAT (; chloramphenicol acetyltransferase) gene in the cloning site of the vector of claim 13.

FIG. 1



MON
BamHI/SalI/HpaI
HindIII
SD
SA
HindIII
NotI/EcoRI
LTR
IRES
NEO
LTR
HHIR
R523Bam
HindIII
BamHI
HindIII
SD
BamHI
LTR
pCRII-5LTR-SD
HindIII/BamHI
HindIII/BamHI fragment
ΔSA
HindIII
SD
BamHI/SalI/HpaI
NotI/EcoRI
LTR
IRES
NEO
LTR
HpaI
XbaI
HindIII
CAT
XbaI/HindIII fragment
Klenow treatment
pC3.1-CAT
ΔSA-CAT
HindIII
SD
NotI/EcoRI
LTR
CAT
IRES
NEO
LTR

FIG. 2

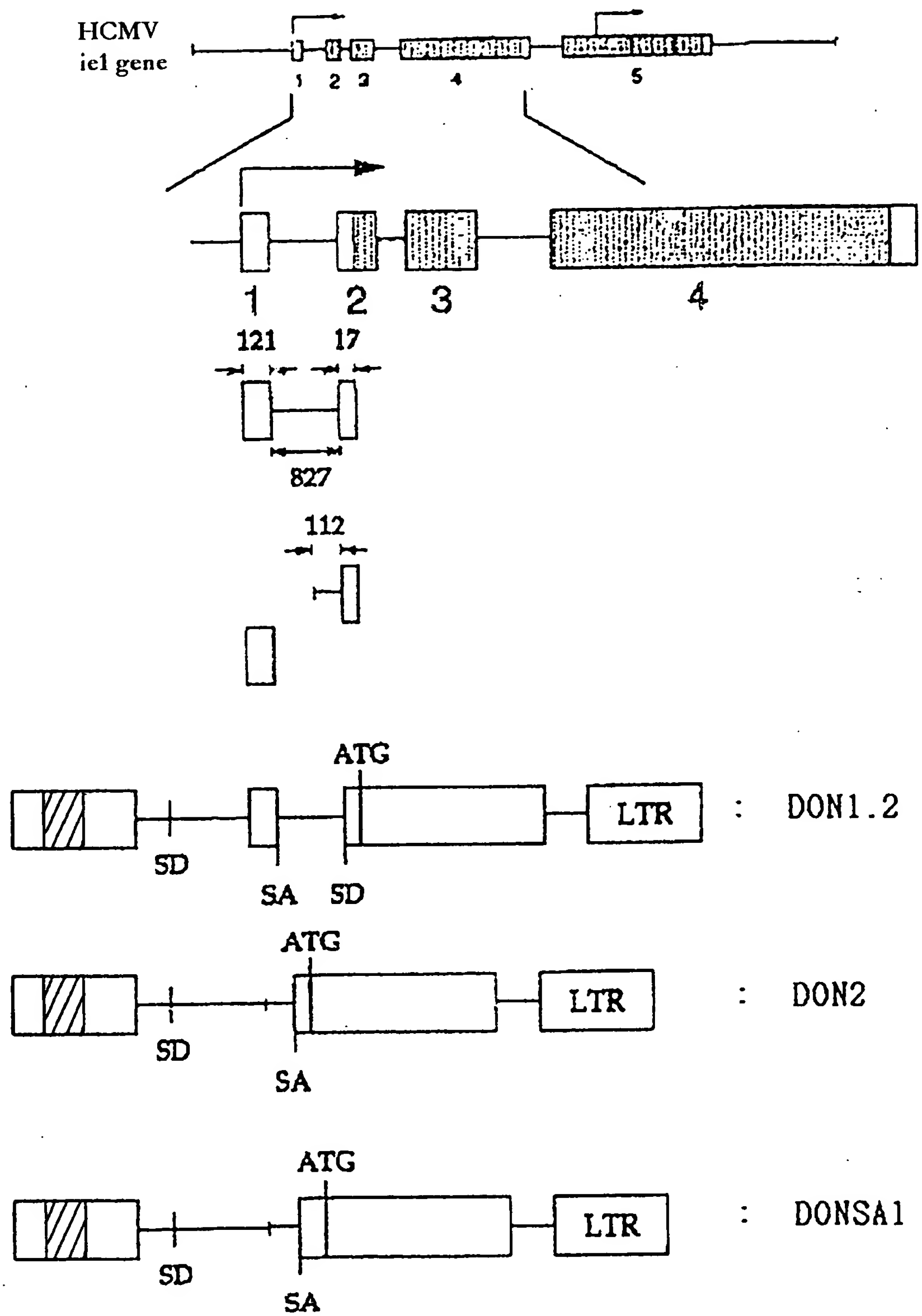

HCMV
ie1 gene
1
2
3
4
5
1
2
3
4
121
17
827
112
ATG
LTR
: DON1.2
SD
SA
SD
ATG
LTR
: DON2
SD
SA
ATG
LTR
: DONSA1
SD
SA

FIG. 3a

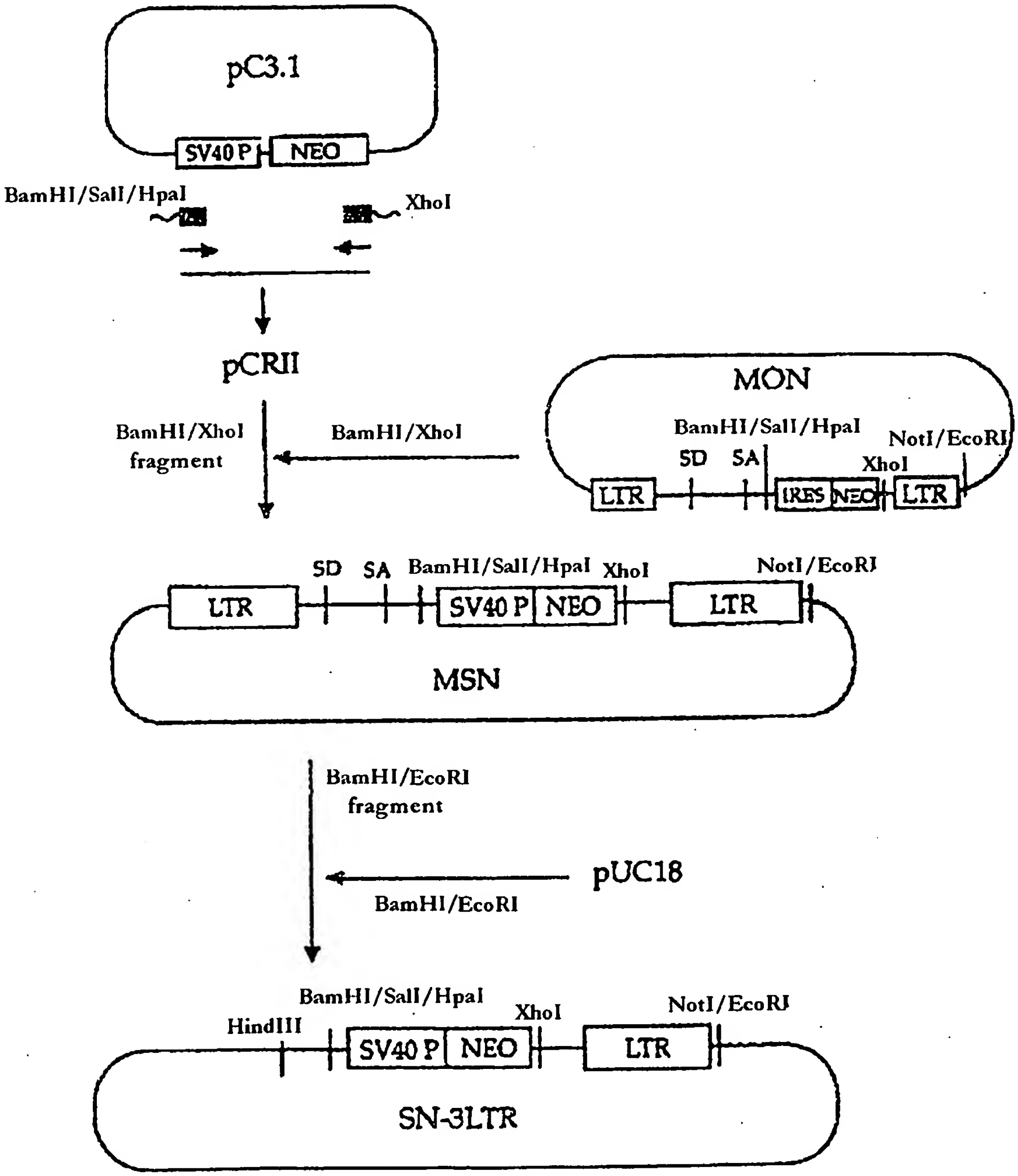

pC3.1
SV40 P
NEO
BamHI/SalI/HpaI
XhoI
pCRII
BamHI/XhoI fragment
BamHI/XhoI
MON
BamHI/SalI/HpaI
NotI/EcoRI
SD
SA
XhoI
LTR
IRES
NEO
LTR
SD
SA
BamHI/SalI/HpaI
XhoI
NotI/EcoRI
LTR
SV40 P
NEO
LTR
MSN
BamHI/EcoRI fragment
pUC18
BamHI/EcoRI
BamHI/SalI/HpaI
XhoI
NotI/EcoRI
HindIII
SV40 P
NEO
LTR
SN-3LTR

FIG. 3b

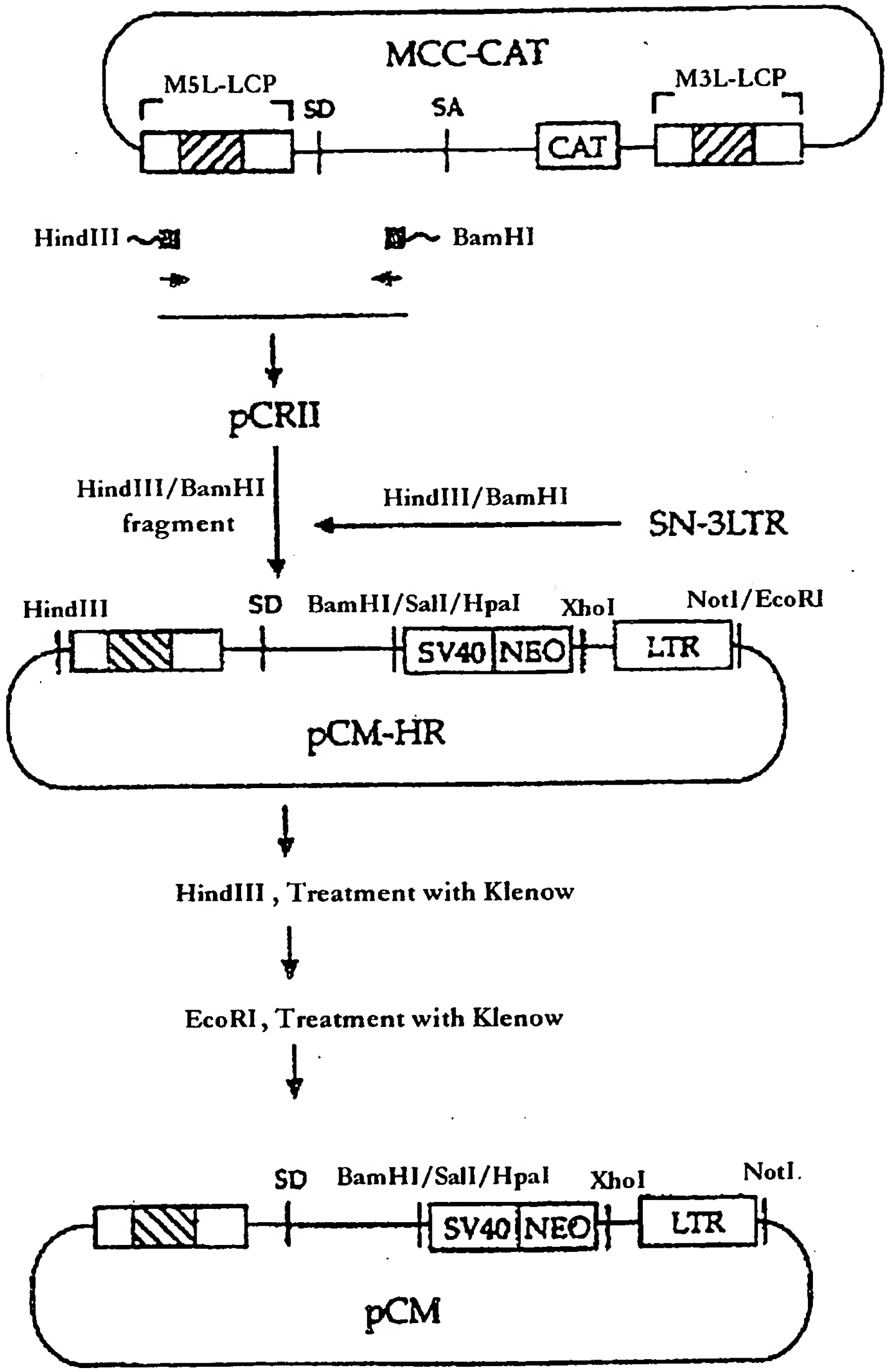
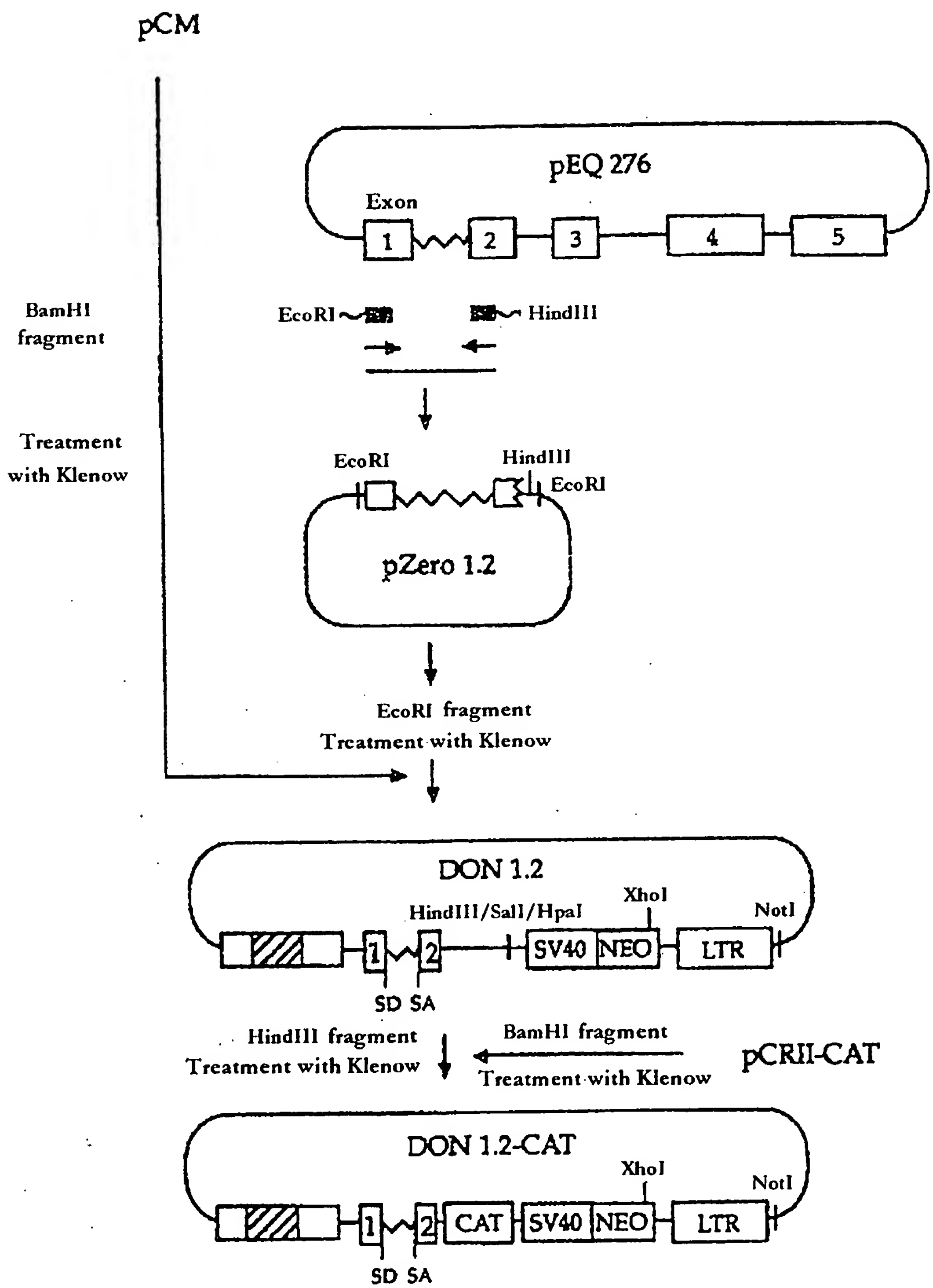

MCC-CAT
M5L-LCP
SD
SA
CAT
M3L-LCP
HindIII
BamHI
pCRII
HindIII/BamHI
fragment
HindIII/BamHI
SN-3LTR
HindIII
SD
BamHI/SalI/HpaI
XhoI
NotI/EcoRI
SV40
NEO
LTR
pCM-HR
HindIII , Treatment with Klenow
EcoRI , Treatment with Klenow
SD
BamHI/SalI/HpaI
XhoI
NotI
SV40
NEO
LTR
pCM

FIG. 4a


pCM
pEQ 276
Exon
1
2
3
4
5
BamHI fragment
EcoRI
HindIII
Treatment with Klenow
EcoRI
HindIII
EcoRI
pZero 1.2
EcoRI fragment
Treatment with Klenow
DON 1.2
XhoI
HindIII/SalI/HpaI
NotI
SV40
NEO
LTR
SD SA
HindIII fragment
Treatment with Klenow
BamHI fragment
Treatment with Klenow
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DON 1.2-CAT
XhoI
NotI
CAT
SV40
NEO
LTR
SD SA

FIG. 4b

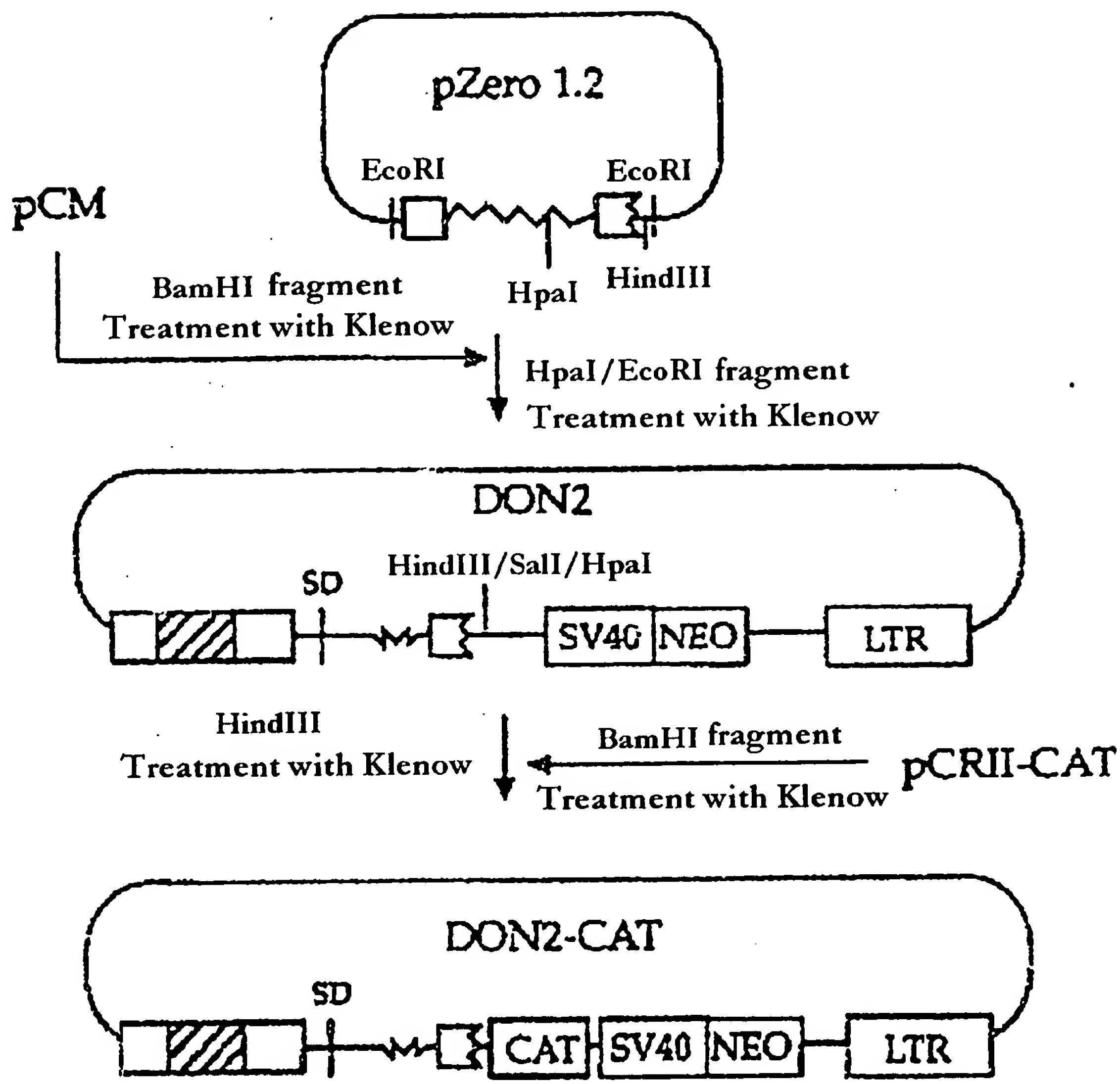
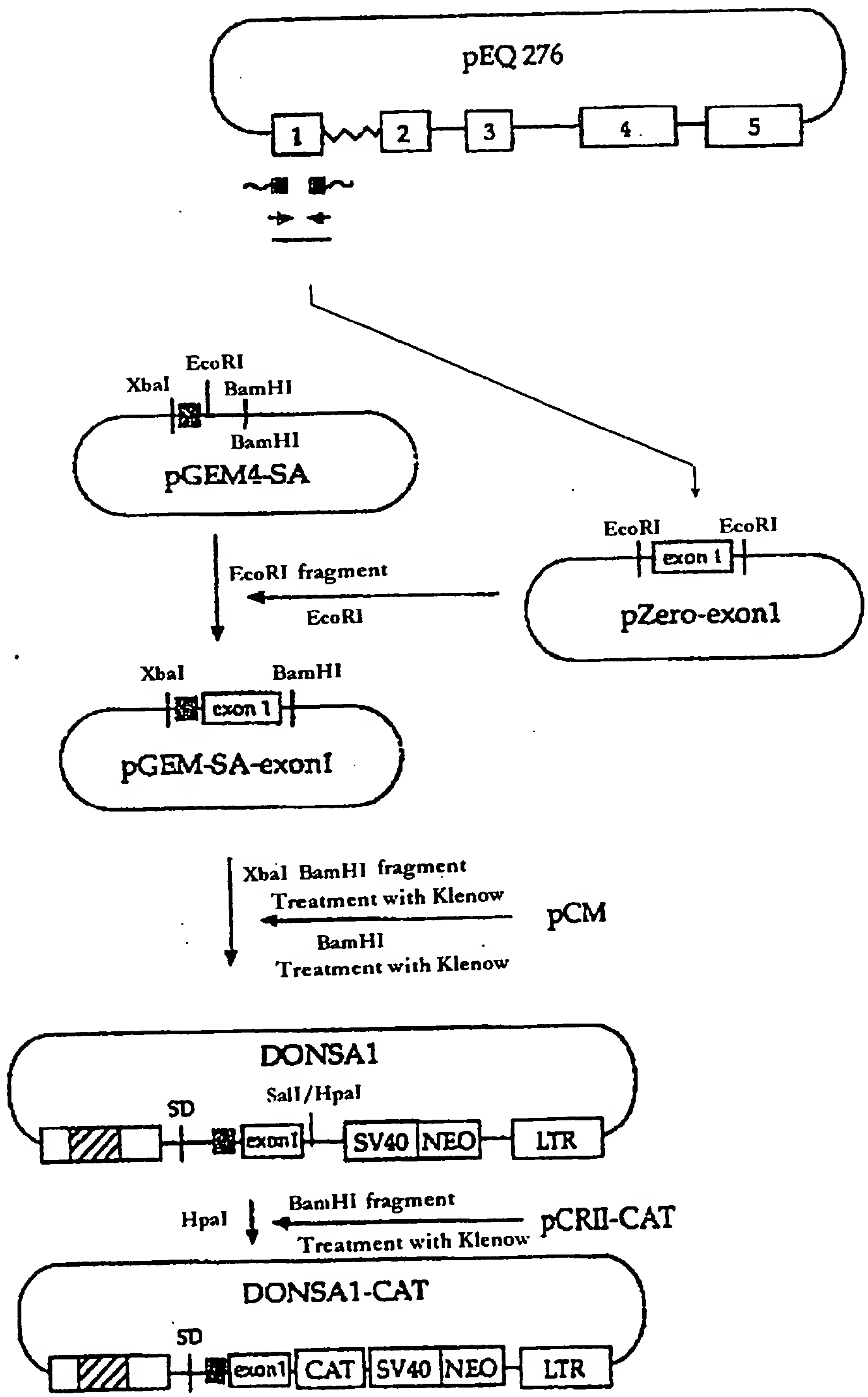

pZero 1.2
EcoRI
EcoRI
pCM
BamHI fragment
Treatment with Klenow
HpaI
HindIII
HpaI/EcoRI fragment
Treatment with Klenow
DON2
SD
HindIII/SalI/HpaI
SV40
NEO
LTR
HindIII
Treatment with Klenow
BamHI fragment
Treatment with Klenow
pCRII-CAT
DON2-CAT
SD
CAT
SV40
NEO
LTR

FIG. 4c



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1
2
3
4
5
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XbaI
BamHI
BamHI
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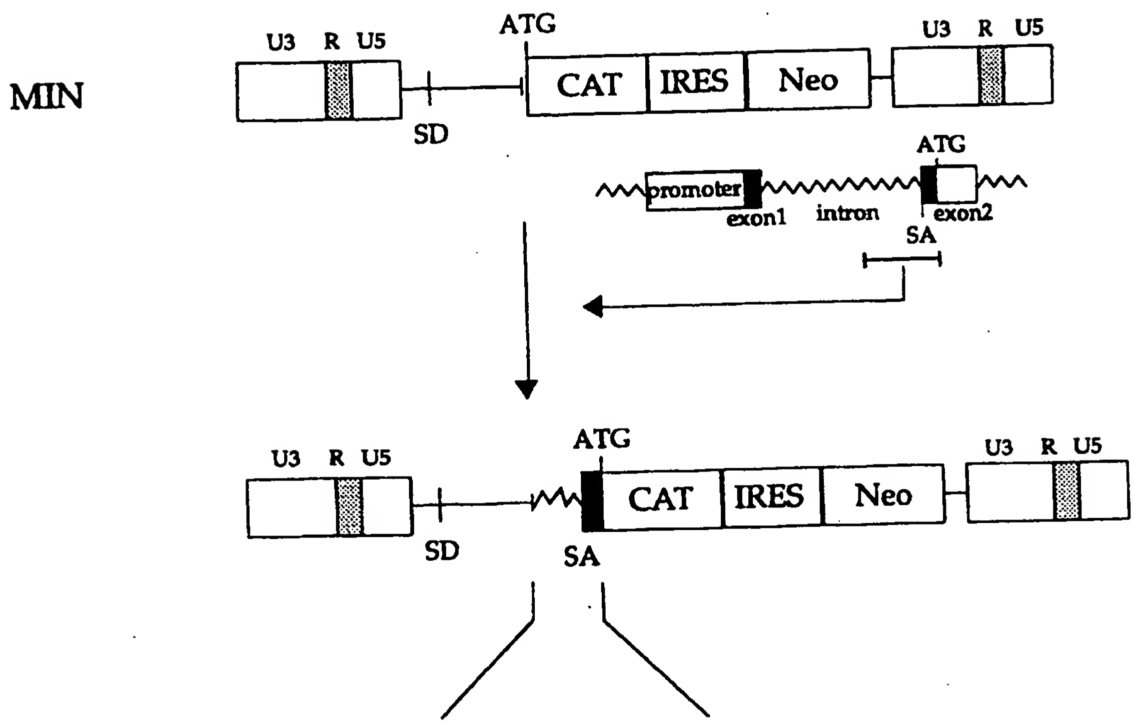
EcoRI
EcoRI
exon 1
pZero-exon1
EcoRI fragment
EcoRI
XbaI
BamHI
exon 1
pGEM-SA-exon1
XbaI BamHI fragment
Treatment with Klenow
BamHI
Treatment with Klenow
pCM
DONSA1
SD
SalI/HpaI
exon1
SV40
NEO
LTR
HpaI
BamHI fragment
Treatment with Klenow
pCRII-CAT
DONSA1-CAT
SD
exon1
CAT
SV40
NEO
LTR

FIG 5



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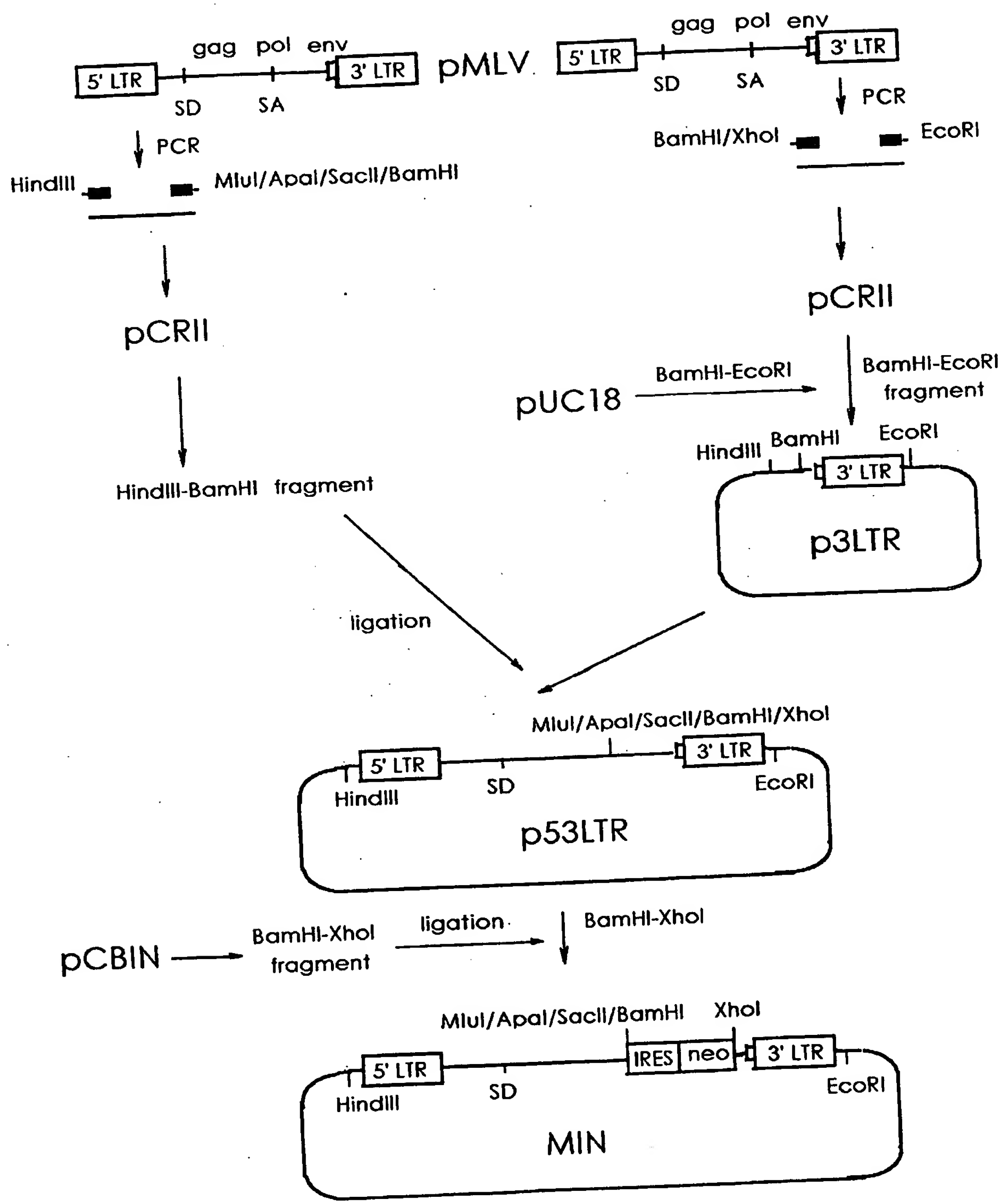
MIN-GI	Human GAPDH gene	(+185 to +317)
MIN-EI	Human EF1-α gene	(+772 to +1008)

FIG 6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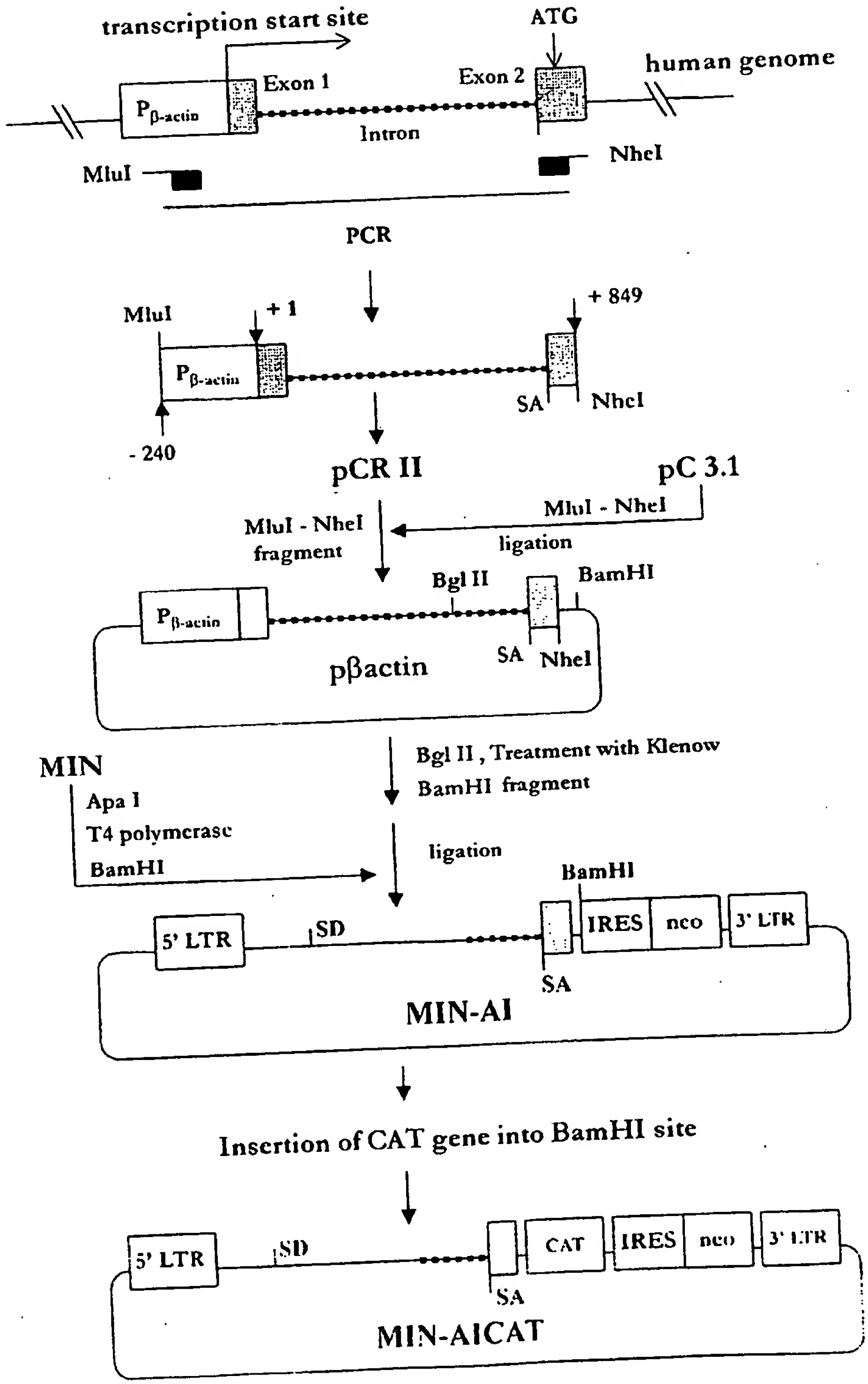
pol
env
5' LTR
3' LTR
SD
SA
pMLV
PCR
HindIII
MluI/ApaI/SacII/BamHI
BamHI/XhoI
EcoRI
pCRII
pCRII
pUC18
BamHI-EcoRI
BamHI-EcoRI fragment
HindIII-BamHI fragment
HindIII
BamHI
EcoRI
p3LTR
ligation
MluI/ApaI/SacII/BamHI/XhoI
HindIII
EcoRI
p53LTR
pCBIN
BamHI-XhoI fragment
ligation
BamHI-XhoI
MluI/ApaI/SacII/BamHI
XhoI
IRES
neo
MIN

FIG 7a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ATG
human genome
Exon 1
Exon 2
$P_{\beta\text{-actin}}$
Intron
MluI
NheI
PCR
MluI
+ 1
+ 849
$P_{\beta\text{-actin}}$
SA
NheI
- 240
pCR II
pC 3.1
MluI - NheI
MluI - NheI
fragment
ligation
Bgl II
BamHI
$P_{\beta\text{-actin}}$
pβactin
SA
NheI
MIN
Apa I
T4 polymerase
BamHI
Bgl II , Treatment with Klenow
BamHI fragment
ligation
BamHI
5' LTR
SD
IRES
neo
3' LTR
SA
MIN-AI
Insertion of CAT gene into BamHI site
5' LTR
SD
CAT
IRES
neo
3' LTR
SA
MIN-AICAT

FIG 7b

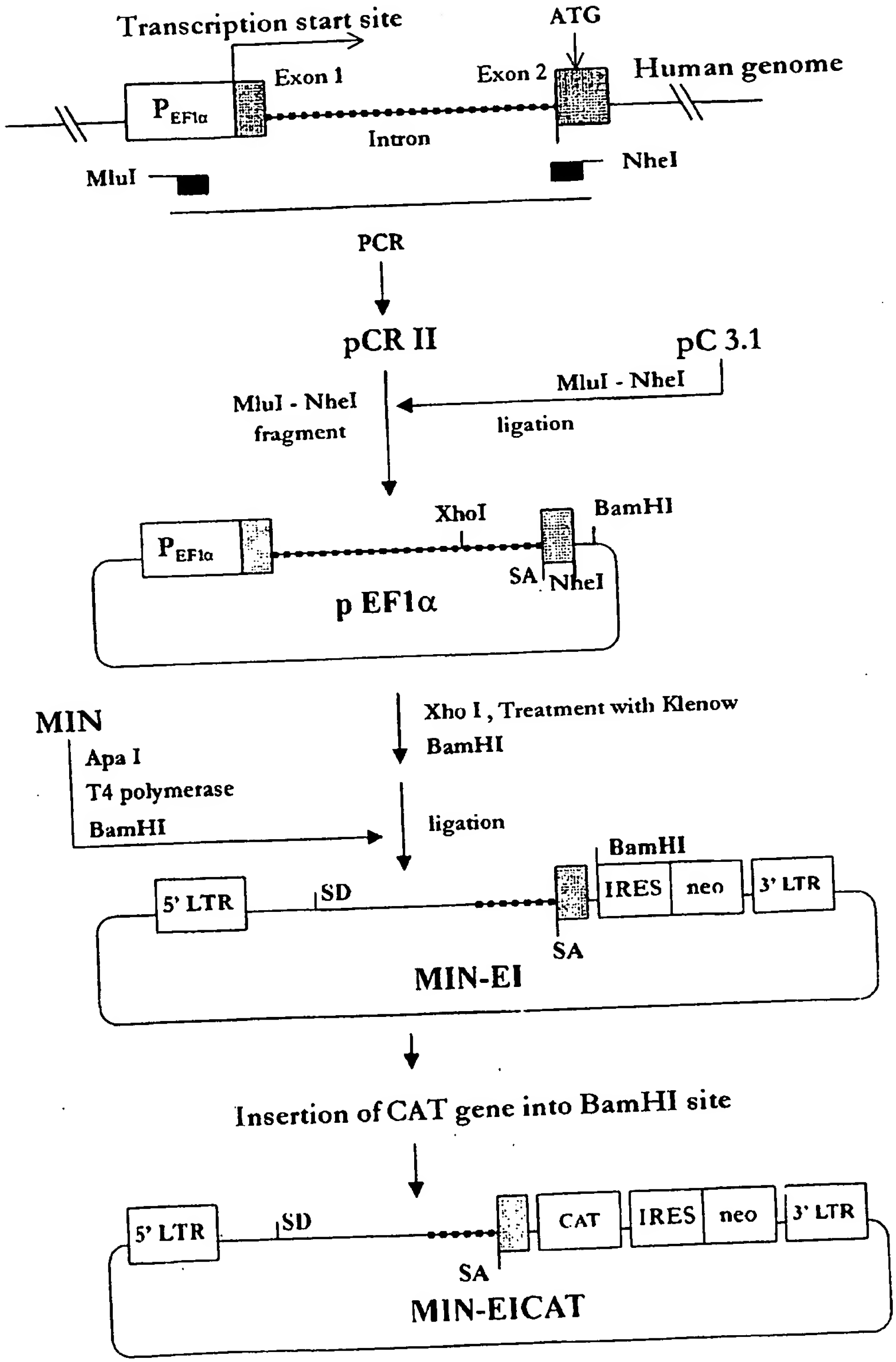
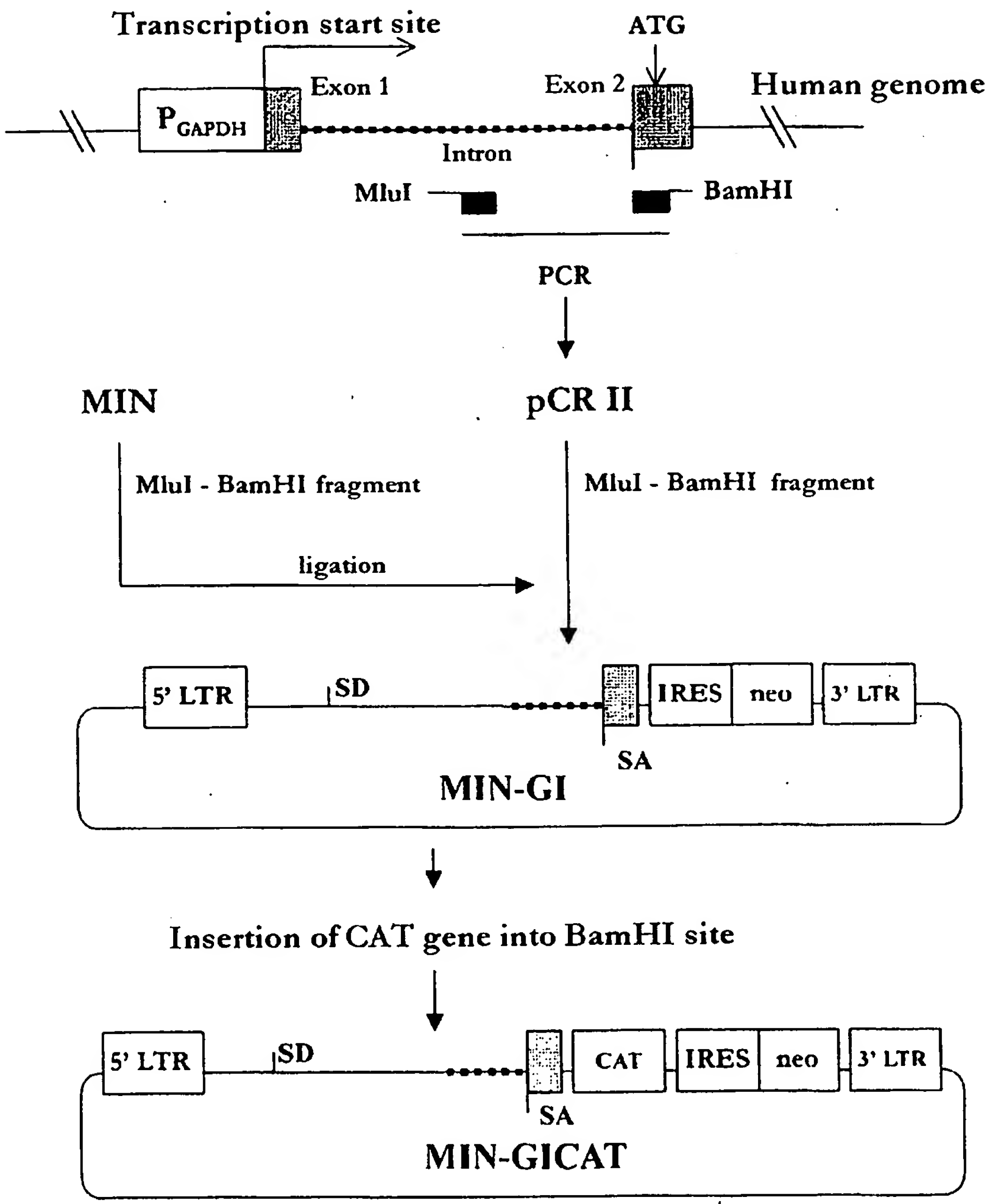

Transcription start site
ATG
Exon 1
Exon 2
Human genome
$P_{EF1\alpha}$
Intron
MluI
NheI
PCR
pCR II
pC 3.1
MluI - NheI
MluI - NheI
fragment
ligation
XhoI
BamHI
$P_{EF1\alpha}$
SA
NheI
p EF1α
MIN
Apa I
T4 polymerase
BamHI
Xho I , Treatment with Klenow
BamHI
ligation
BamHI
5' LTR
SD
IRES
neo
3' LTR
SA
MIN-EI
Insertion of CAT gene into BamHI site
5' LTR
SD
CAT
IRES
neo
3' LTR
SA
MIN-EICAT

FIG 7c


Transcription start site
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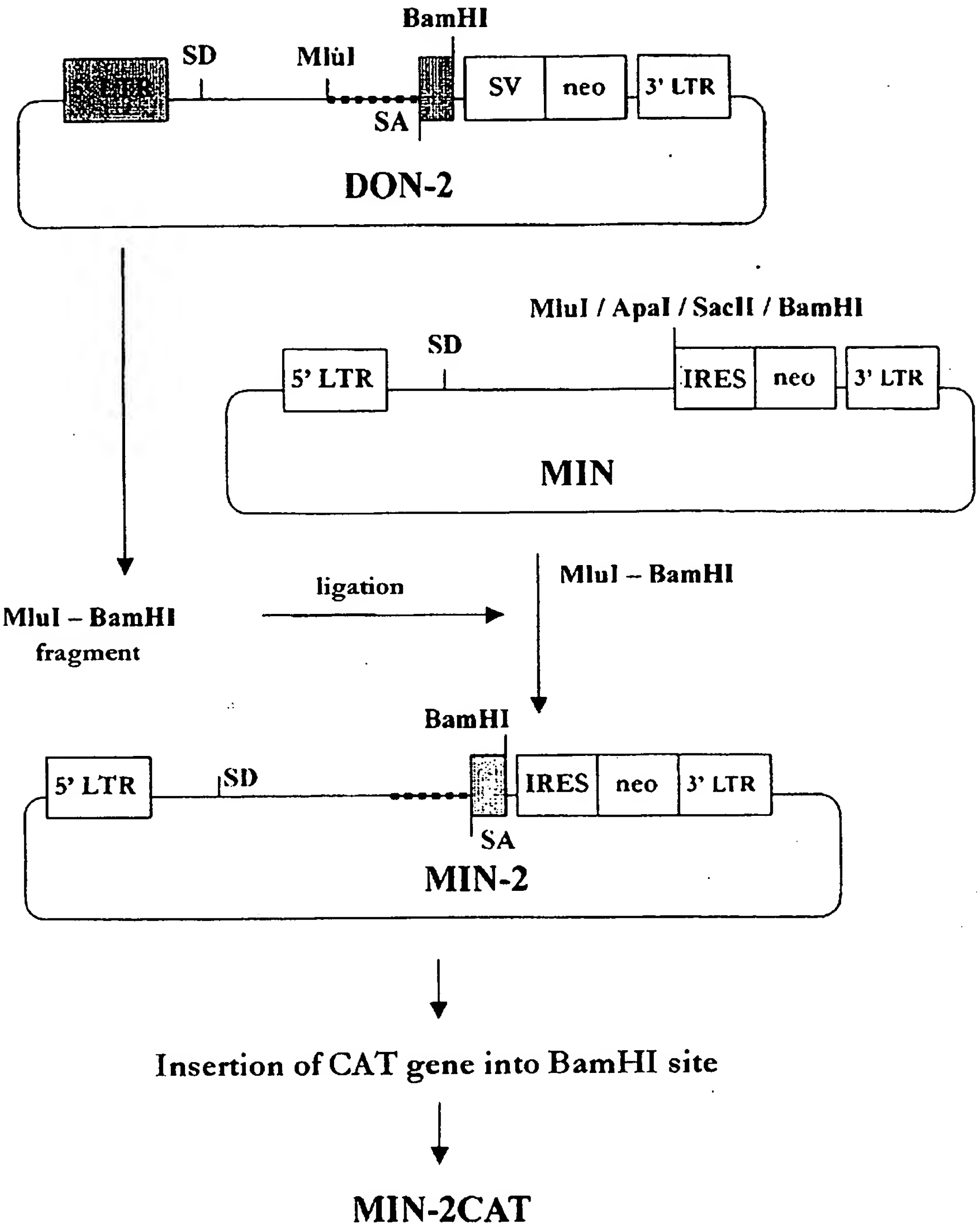
Exon 1
Exon 2
Human genome
PGAPDH
Intron
MluI
BamHI
PCR
MIN
pCR II
MluI - BamHI fragment
MluI - BamHI fragment
ligation
5' LTR
SD
IRES
neo
3' LTR
SA
MIN-GI
Insertion of CAT gene into BamHI site
5' LTR
SD
CAT
IRES
neo
3' LTR
SA
MIN-GICAT

FIG 7d


BamHI
SD
MluI
5' LTR
SV
neo
3' LTR
SA
DON-2
MluI / ApaI / SacII / BamHI
SD
5' LTR
IRES
neo
3' LTR
MIN
ligation
MluI – BamHI
MluI – BamHI
fragment
BamHI
5' LTR
SD
IRES
neo
3' LTR
SA
MIN-2
Insertion of CAT gene into BamHI site
MIN-2CAT

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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. Sunyoung Kim
HanShin Su-re A.P.T 3-805
BanPo-Dong
Seoul, Korea

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR :	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Top10-DONSA1	KCCM-10127

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☐ a scientific description

☐ a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on June. 5. 1998 (date of the original deposit)[1]

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name : Korean Culture Center of Microorganisms	Signature(s) of person(s) having the power to represent the International Depositary Authority of of authori[illegible]
Address : Department of Food Engineering College of Eng. Yonsei University Sodaemun-gu, Seoul 120-749 Korea	Date: June. 12. 1998

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

Form BP/4 (KCCM Form 17)

Sole page

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. Sunyoung Kim
HanShin Su-re A. P. T 3 805
BanPo Dong
Seoul, Korea

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : Top10-DON2	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM-10128

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☐ a scientific description

☐ a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authouity accepts the microorganism identified under I above, which was received by it on June. 5. 1998 (date of the original deposit)[1]

IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Department of Food Engineering College of Eng. Yonsei University Sodaemun-gu, Seoul 120-749 Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of of authorized official(s): Date: June. 12. 1998

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authouity.

Form BP/4 (KCCM Form 17)

Sole page

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. Sunyoung Kim
3-805 Hanshin Seorae Apartment,
Banpo-Dong, Seocho-Ku,
Seoul 137-040,
Korea

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR :	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
MIN-EICAT(Top10)	KCCM-10163

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☐ a scientific description

☐ a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authouity accepts the microorganism identified under I above, which was received by it on June. 2. 1999 (date of the original deposit)[1]

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name : Korean Culture Center of Microorganisms Address : 361-221, Yurim B/D Hongje-1-dong, Seodaemun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of of authouized official(s) : Date: June. 9. 1999.

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authouity.

Form BP/4 Sole page

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE.

INTERNATIONAL FORM

To. Sunyoung Kim
3-805 Hanshin Seorae Apartment,
Banpo-Dong, Seocho-Ku,
Seoul 137-040,
Korea

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR :	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
MIN-2CAT(Top10)	KCCM-10164

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☐ a scientific description

☐ a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authouity accepts the microorganism identified under I above, which was received by it on June. 2. 1999 (date of the original deposit)[1]

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name : Korean Culture Center of Microorganisms Address : 361-221, Yurim B/D Hongje-1-dong, Seodaemun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of of authouized official(s) : Date: June. 9. 1999.

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired : where a deposit made outside the Budapest Treaty after the acquisition of the status of i nternational depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authouity.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00334

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 N 15/867, 15/11, 5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 N 15/867, 15/11, 5/10

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98/12338 A1 (VIROMEDICA PACIFIC LIMITED) 26 March 1998 (26.03.98), claims 1, 2, 6-9, 12-14, 16.	1-8, 10,11
A	US 5 688 688 A (LUCIW et al.) 18 November 1997 (18.11.97), claims 1,2.	1,4,5,8,9
A	WO 94/24298 A1 (INSTITUT PASTEUR) 27 October 1994 (27.10.94), abstract.	1
A	WO 95/22617 A1 (UNIVERSITE PIERRE ET MARIE CURIE), 24 August 1995 (24.08.95), abstract; claims 1, 7.	1
A	TCHENIO et al. High-frequency intracellular transposition of a defective mammalian provirus defected by an in situ colorimetic assay, J. Virol. 1992, 66(3), 1571-8 (Eng.). Columbus, Ohio, USA: Chemical abstracts, Vol. 116, No. 17, 27 April 1992 (27.04.92), page 191, left column, the abstract No. 167408q.	1

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
„A" document defining the general state of the art which is not considered to be of particular relevance
„E" earlier application or patent but published on or after the international filing date
„L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
„O" document referring to an oral disclosure, use, exhibition or other means
„P" document published prior to the international filing date but later than the priority date claimed
„T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
„X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
„Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 October 1999 (01.10.99)	08 November 1999 (08.11.99)

Name and mailing adress of the ISA/AT	Authorized officer
Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/200	Mosser Telephone No. 1/53424/437

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR 99/00334

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche		Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets		Datum der Veröffentlichung Publication date Date de publication
WO A1	9812338	26-03-1998	AU A1	44012/97	14-04-1998
			GB A0	9810991	22-07-1998
			GB A1	2322131	19-08-1998
			JP T2	11501223	02-02-1999
US A	5688688	18-11-1997	AT E	90384	15-06-1993
			DE C0	3587394	15-07-1993
			DE T2	3587394	05-01-1994
			EP A1	181150	14-05-1986
			EP A1	518443	16-12-1992
			EP B1	181150	09-06-1993
			HK A	1024/95	30-06-1995
			JP A2	61173782	05-08-1986
			JP A2	7311199	28-11-1995
			JP A2	7313184	05-12-1995
			JP A2	9235297	09-09-1997
			JP B2	2865283	08-03-1999
			US A	5156949	20-10-1992
WO A1	9424298	27-10-1994	AU A1	66812/94	08-11-1994
			AU B2	688321	12-03-1998
			AU A1	59675/98	04-06-1998
			AU B2	704371	22-04-1999
			EP A1	702723	27-03-1996
			FR A1	2708202	03-02-1995
			FR B1	2708202	29-03-1996
			JP T2	8508880	24-09-1996
			US A	5906817	25-05-1999
			FR A1	2704236	28-10-1994
			FR B1	2704236	23-06-1995
WO A1	9522617	24-08-1995	AT E	172496	15-11-1998
			AU A1	18518/95	04-09-1995
			CA AA	2183151	24-08-1995
			DE C0	69505493	26-11-1998
			DE T2	69505493	22-04-1999
			EP A1	738327	23-10-1996
			EP A2	855185	29-07-1998
			EP B1	738327	21-10-1998
			EP A3	855185	18-11-1998
			ES T3	2124532	01-02-1999
			FI A0	963246	19-08-1996
			FI A	963246	16-10-1996
			FR A1	2716459	25-08-1995
			FR B1	2716459	10-05-1996
			IL A0	112736	26-05-1995
			JP T2	9509060	16-09-1997
			NO A0	963358	13-08-1996
			NO A	963358	02-09-1996
			US A	5948675	07-09-1999
			FR A1	2716461	25-08-1995
			ZA A	9501451	18-12-1995